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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C F R. § 1 53(b))

Attorney Docket No.	UF-221C1XC1
First Inventor or Application Identifier	Moyer, Richard W.
Title	Materials and Methods For Delivery And Expression of Heterologous ... Cells
Express Mail Label No	EK841464075US

JC926 U.S. PTO 09/662254 09/14/00

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: **Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231**

1. [X] * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original and a duplicate for fee processing)
2. [X] Specification [Total Pages 92]
(preferred arrangement set forth below)
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings (if filed)
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. [X] Drawing(s) (35 U.S.C. 113) [Total Sheets 10]
(two additional sets)
4. Oath or Declaration [Total Pages 2]
   a. [X] Newly executed (original or copy) unsigned
   b. [ ] Copy from a prior application (37 C.F.R. § 1.63(d))
   (for continuation/divisional with Box 16 completed)
      i. [ ] DELETION OF INVENTOR(S)
      Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

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5. [ ] Microfiche Computer Program (Appendix)
6. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
   a. [X] Computer Readable Copy
   b. [X] Paper Copy (identical to computer copy)
   c. [X] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. [ ] Assignment Papers (cover sheet & document(s))
8. [ ] 37 C.F.R.§3.73(b) Statement (when there is an assignee) [ ] Power of Attorney
9. [ ] English Translation Document (if applicable)
10. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
11. [ ] Preliminary Amendment
12. [X] Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
13. [X] * Small Entity Statement(s) (PTO/SB/09-12) [ ] Statement filed in prior application, Status still proper and desired
14. [ ] Certified Copy of Priority Document(s) (if foreign priority is claimed)
15. [X] Other: Petition under 37 CFR 1.84(2) w/3 sets of color figures
Claims benefit to USSN 60/224,479 filed 8/10/2000

16. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:
[ ] Continuation [ ] Divisional [X] Continuation-in-part (CIP) of prior application No. 09 / 086,651
Prior application information: Examiner Beckerleg Group / Art Unit: 1632

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

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Signature	David Saliwanchik	Date	9-14-00

DOCKET NO. UF-221C1XC1

JC926 U.S. PTO 09/662254 09/14/00

FEE TRANSMITTAL FORM

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Sir:

Transmitted herewith for filing is the patent application of:

Inventor(s): Richard W. Moyer, Yi Li, Alison L. Bawden

Entitled: Materials And Methods For Delivery And Expression Of Heterologous DNA In Vertebrate Cells

- ■ A Utility Patent Application Transmittal Form accompanies this Fee Transmittal Form.
- ■ The filing fee is calculated below:

CLAIMS AS FILED

	Number filed		Number Extra	Rate	Fee
Basic Fee					$ 345.00
Total Claims	75	– 20 =	55	x $09	495.00
Independent Claims	7	– 3 =	4	x $39	156.00
Presentation of Multiple Dependent Claim(s) ($165)					0
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Sept. 14, 2000
Date

David Saliwanchik
Attorney of Record

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Applicant or Patentee: Richard W. Moyer, Yi Li, Alison L. Bawden
Attorney's Docket No. UF-221C1XC1
Serial or Patent No.:
Filed or Issued:
For: Materials And Methods For Delivery And Expression Of Heterologous DNA In Vertebrate Cells

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9 (f) and 1.27 (c)) – NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION University of Florida
ADDRESS OF ORGANIZATION 223 Grinter Hall
Gainesville, FL 32611

TYPE OF ORGANIZATION

[X] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a)(3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE ______)
(CITATION OF STATUTE ______)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE ______)
(CITATION OF STATUTE ______)

I hereby declare that the above identified nonprofit organization qualifies as a nonprofit organization as defined in 37 CFR 1.9 (d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, with regard to the invention described in the above-identified:

[ ] PATENT [X] APPLICATION

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization identified above with regard to the above-identified invention.

If the rights held by the above identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9 (d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9 (e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring their status as small entities. (37 CFR 1.27)

NAME ______
ADDRESS ______
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME ______
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[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change of status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Thomas E. Walsh, Ph.D.
TITLE IN ORGANIZATION Director of Sponsored Research
ADDRESS OF PERSON SIGNING 223 Grinter Hall
Gainesville, FL 32611

SIGNATURE TWalsh DATE September 14, 2000

DESCRIPTION

MATERIALS AND METHODS FOR DELIVERY AND EXPRESSION OF HETEROLOGOUS DNA IN VERTEBRATE CELLS

The subject invention was made with government support under a research project supported by U.S. Department of Agriculture Grant No. 97-35302-4431 and National Institute of Health Grant No. P50-HL59412-01. The government has certain rights in this invention.

Cross-Reference to a Related Application

This application is a continuation-in-part of co-pending application Serial No. 09/086,651, filed May 29, 1998. This application also claims the benefit of provisional patent application Serial No. 60/224,479; filed August 10, 2000.

Background of the Invention

Gene therapy is a powerful concept just now beginning to see applications designed to treat human diseases such as genetic disorders and cancer. The introduction of genes into an organism can be achieved in a variety of ways, including virus-based vectors. Viral gene therapy vectors can either be designed to deliver and express genes permanently (stable integration of a foreign gene into host chromosome) or transiently (for a finite period of time).

Current virus-based gene transfer vectors are typically derived from animal viruses, such as retroviruses, herpesviruses, adenoviruses, or adeno-associated viruses. Generally, these viruses are engineered to remove one or more genes of the virus. These genes may be removed because they are involved in viral replication and/or to provide the capacity for insertion and packaging of foreign genes. Each of these known vectors has some unique advantages as well as disadvantages. One primary disadvantage is an inability to readily package and deliver large DNA inserts that are greater than 10 kb in size.

To illustrate the problem of capacity of most gene therapy vectors, one need only consider adeno-associated virus (AAV), one of the most promising of the gene therapy vectors. Adeno-associated virus (AAV) is a parvovirus which consists of a 4.7 kb single stranded DNA genome (Nienhuis, A.W., C.E. Walsh, J.M. Liu [1993] "Viruses as therapeutic gene transfer vectors" In: N.S. Young (ed.) *Viruses and Bone Marrow,* Marcel Decker, New York, pp. 353-414). The viral genome consists of the family of *rep* genes responsible for regulatory function and DNA replication and the *cap* genes that encode the capsid proteins. The AAV coding region is flanked by 145 nucleotide inverted terminal repeat (ITR) sequences which are the minimum cis-acting elements essential for replication and encapsidation of the genome. In the absence of a helper virus such as adenovirus, AAV causes a latent infection characterized by the integration of viral DNA into the cellular genome. The major advantages of recombinant AAV (rAAV) vectors include a lack of pathogenicity in humans (Berns, K.I. and R.A. Bohenzky [1987] "Adeno-associated viruses: an update" *Adv. Virus Res.* 32:243-306), the ability of wild-type AAV to integrate stably into the long arm of chromosome 19 (Kotin, R.M., R.M. Linden, K.I. Berns [1992] "Characterization of a preferred site on human chromosome 10q for integration of adeno-associated virus DNA by nonhomologous recombination" *EMBO J* 11:5071-5078), the potential ability to infect nondividing cells (Kaplitt *et al.* [1994] "Long term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain" *Nat. Genet.* 8:148-154), and broad range of infectivity. However, the packaging capacity of AAV limits the size of the inserted heterologous DNA to about 4.7 kb. Gene therapy vector systems are also needed that combine a large carrying capacity with high transduction efficiency *in vivo.*

Until recently, complex insect viruses (entomoviruses) had not been considered for use as possible viral gene therapy vectors. In the past, studies of entomoviruses have mainly concentrated on their use as biopesticides, expression systems or taxonomic novelties to compare to their mammalian virus counterparts.

The family *Poxviridae* comprises two subfamilies, the Chordopoxviridae (vertebrate) and the Entomopoxviridae (insect) viruses (EPVs). EPVs were first discovered in the early

1960's, and have subsequently been shown to have a worldwide distribution. The subfamily contains three genera; A, B and C, which infect beetles, moths (lepidoptera) and grasshoppers, and midge flies respectively (Moyer, R.W. [1994] Entomopoxviruses, p. 392-397, <u>Encyclopedia of Virology</u>, R.G. Webster and A. Granoff (eds.), Academic Press Ltd, London). It should be recognized that classification within the three EPV genera is based solely on morphological and host range criteria and not molecular properties. Indeed, it is now clear that the group B viruses of butterflies and moths (lepidoptera) and grasshoppers (orthoptera) are quite distinct from one another (Afonso, C.L., E.R. Tulman, Z. Lu, E. Oma, G.F. Kutish, and D.L. Rock [1999] "The genome of *Melanoplus sanguinipes* Entomopoxvirus" *J. Virol.* 73:533-552). AmEPV was originally isolated in India from the red hairy caterpillar, and it is the prototype virus of this group. This is primarily because of its ability to be easily grown in cultured insect cells, although certain *Choristoneura* and *Heliothis* EPVs have also been shown to replicate in cell cultures at low levels (Fernon, C.A., A.P. Vera, R. Crnov, J. Lai-Fook, R.J. Osborne, and D.J. Dall [1995] "Replication of *Heliothis armigera* entomopoxvirus *in vitro*" *J. Invertebr. Pathol.* **66**:216-223; Lytvyn, V., Y. Fortin, M. Banville, B. Arif, and C. Richardson [1992] "Comparison of the thymidine kinase genes from three entomopoxviruses" *J. Gen. Virol.* 73:3235-3240).

EPVs are the most distant relatives of mammalian poxviruses and exhibit both similarities and differences to the more commonly studied chordopoxviruses, such as vaccinia virus (VV). Similarities include morphology, a large linear double stranded genome (previously estimated at 225 kb for AmEPV, 190 kb for VV), common transcriptional regulation sequence motifs, non-spliced transcripts and a cytoplasmic site of replication. Differences include the G+C content of the viral DNA (a low 18% for AmEPV, 37% for VV), optimal growth temperatures (28°C for AmEPV, 37°C for VV), and host range. AmEPV does not replicate in vertebrate cells, and VV does not replicate in insect cells, although both viruses enter their respective non-permissive cells and initiate a replicative cycle (Langridge, W.H. [1983] "Detection of *Amsacta moorei* entomopoxvirus and vaccinia virus proteins in cell cultures restrictive for poxvirus multiplication" *J. Invertebr. Pathol.* **42**:77-82).

Generally, growth of AmEPV in insect cell cultures is similar to that of vertebrate poxviruses in mammalian cells. Receptors mediating poxvirus attachment and entry appear to be widespread and common, as EPVs infect vertebrate cells and VV infects insect cells (Li, Y., R.L. Hall, and R.W. Moyer [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562; Li, Y., S. Yuan, and R.W. Moyer [1998] "The non-permissive infection of insect (gypsy moth) LD-652 cells by vaccinia virus" *Virology* 248:74-82). It is assumed by analogy with the vertebrate poxviruses that AmEPV gene expression patterns can be classified as early, intermediate and late, but experimental data is minimal (Winter, J., R.L. Hall, and R.W. Moyer [1995] "The effect of inhibitors on the growth of the entomopoxvirus from *Amsacta moorei* in *Lymantria dispar* (gypsy moth) cells" *Virology* 211:462-473). EPVs have been shown to contain vertebrate poxvirus promoter elements and early transcription termination motifs (Afonso, C.L., E.R. Tulman, Z. Lu, E. Oma, G.F. Kutish, and D.L. Rock [1999] "The genome of *Melanoplus sanguinipes* Entomopoxvirus" *J. Virol.* 73:533-552; Hall, R.L. and R.W. Moyer [1991] "Identification, cloning, and sequencing of a fragment of *Amsacta moorei* entomopoxvirus DNA containing the spheroidin gene and three vaccinia virus-related open reading frames" *J. Virol.* 65:6516-6527). The most unique feature of poxvirus replication is development mostly, if not exclusively, within the cytoplasm. As a consequence of cytoplasmic development, EPV promoters (like those of vertebrate poxviruses) are recognized only by the virally encoded transcription system. The general availability of poxvirus specific promoters, coupled with exclusion of the nuclear transcription apparatus are major advantages for engineering and control of foreign gene expression related to gene therapy applications.

EPVs, like VV, contain a number of genes which are nonessential for growth in cell culture. Two examples are the thymidine kinase (TK) and spheroidin genes. The spheroidin gene can be viewed as a counterpart to the polyhedrin and A-type (ATI) occlusion genes of baculoviruses and cowpox viruses respectively. VV also contains an ATI gene, but it is defective. Spheroidin is the most abundantly expressed AmEPV gene, and serves to "occlude" infectious virions within an environmentally resistant occlusion body. Both the

AmEPV TK and spheroidin gene can readily serve as sites for insertion and expression of foreign genes by utilizing standard plasmid-mediated recombination.

Entomopoxvirus (EPVs) productively infect and kill only insects (Granados, R.R. [1981] "Entomopoxvirus infections in insects," in *Pathogenesis of Invertebrate Microbial Disease,* p. 102-126, Davidson, E.W. (ed.) New Jersey, Allanheld Totowa) and can be isolated from *Amsacta moorei* (AmEPV), the red hairy caterpillar. Entomopox viruses and vectors have been described (See, for example, U.S. Patent Nos. 5,721,352 and 5,753,258, the disclosure of which is incorporated herein by reference). Like other EPVs, AmEPV cannot productively infect vertebrate cells. Indeed, following addition of AmEPV to vertebrate (mouse L-929) cells at multiplicities up to 10 particles/cell, no changes in cellular morphology (as judged by phase contrast microscopy) are detected (Langridge, W.H. [1983] "Detection of *Amsacta moorei* entomopoxvirus and vaccinia virus proteins in cell cultures restrictive for poxvirus multiplication" *J. Invertebr. Pathol.* 42:77-82).

AmEPV infects vertebrate cells in a non-cytocidal manner and the infection is abortive. Like all poxviruses, the virus is cytoplasmic and does not normally enter the nucleus. A consequence of this unusual biology, is that all poxvirus mediated gene expression takes place in the cytoplasm in the infected cell. AmEPV promoters and those of the eucaryotic cell are completely different and cellular promoters are not recognized by the AmEPV transcription machinery nor are AmEPV viral promoters recognized by RNA polymerase II of the host cell.

Brief Summary of the Invention

The subject invention concerns a novel viral vector system for gene therapy based on an insect poxvirus designed to deliver genes for integration and stable, permanent expression in vertebrate cells. In an exemplified embodiment, a recombinant AmEPV vector was constructed that contains heterologous genes under the control of promoters that the drive the expression of the heterologous genes in vertebrate cells. The *gfp* gene and the gene encoding G418 resistance were used in an exemplified construct. The recombinant AmEPV was used to infect vertebrate cells and following infection the cells were transferred to media

containing G418. Cells expressing both GFP and G418 resistance were obtained. Thus, the vectors of the subject invention can be used to deliver large DNA segments for the engineering of vertebrate cells.

The subject invention also concerns cells that have been infected with or transformed with a recombinant vector of the present invention. The subject invention also concerns methods for providing gene therapy for conditions or disorders of an animal requiring therapy, such as genetic deficiency disorders.

In addition, the subject invention concerns novel AmEVP polypeptides and the polynucleotide sequences which encode these polypeptides. The AmEPV polynucleotide sequences of the subject invention encode a triacylglyceride lipase (SEQ ID NO: 1), a Cu^{++}/Zn^{++} superoxide dismutase (SOD) (SEQ ID NO: 2), a CPD photolyase (SEQ ID NO: 3), a baculovirus-like inhibitor of apoptosis (IAP) (SEQ ID NO: 4), two poly(A) polymerase small subunits (SEQ ID NOS: 5 and 6), two DNA polymerases (SEQ ID NOS: 7 and 8), an ABC transporter-like protein (SEQ ID NO: 9), a Kunitz-motif protease inhibitor (KPI) (SEQ ID NO: 10), and a poly(A) polymerase large subunit (SEQ ID NO: 11).

In addition, the subject invention concerns isolated AmEPV polypeptides encoded by the polynucleotide sequences of the subject invention, including a triacylglyceride lipase (SEQ ID NO: 12), a Cu^{++}/Zn^{++} superoxide dismutase (SOD) (SEQ ID NO: 13), a CPD photolyase (SEQ ID NO: 14), a baculovirus-like inhibitor of apoptosis (IAP) (SEQ ID NO: 15), two poly(A) polymerase small subunits (SEQ ID NOS: 16 and 17), two DNA polymerases (SEQ ID NOS: 18 and 19), an ABC transporter-like protein (SEQ ID NO: 20), a Kunitz-motif protease inhibitor (KPI) (SEQ ID NO: 21), a poly(A) polymerase large subunit (SEQ ID NO: 22) and other AmEPV polypeptides.

The subject invention further pertains to other entomopoxvirus sequences. Polynucleotides of the subject invention include, for example, sequences identified in the attached sequence listing, as well as the tables and figures and described by open reading frame position within the genome.

In addition, the subject invention includes polynucleotides which hybridize with other polynucleotides of the subject invention.

Polynucleotide sequences of this invention have numerous applications in techniques known to those skilled in the art of molecular biology having the benefit of the instant disclosure. These techniques include their use as insertion sites for foreign genes of interest, hybridization probes, for chromosome and gene mapping, in PCR technologies, and in the production of sense or antisense nucleic acids.

Brief Description of the Drawings

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawings(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1 shows a physical map of an exemplified recombinant vector of the subject invention (pAmEPV TKUF5) in which a portion of the plasmid pTKUF5 has been cloned within the AmEPV TK gene flanking regions. TR is the AAV terminal repeat; pA is a polyadenylation site; SD/SA is the SV40 late splice donor, splice acceptor sequence. GFP, the green fluorescent protein gene, is under the control of a CMV promoter. Neo, the neomycin resistance gene, is under the control of a herpes TK gene promoter.

Figure 2 shows an electrophoretic analysis of transformed mammalian cell lines. Each lane contains *Hind*III digested genomic DNA. Lane P contains genomic DNA from 293 cells and pTR-UF5 plasmid, as a positive control. Lanes A1 through A5 contain DNA extracted from transformed cell lines made by recombinant AmEPV (AmEPVpTKUF5) infection. Lanes B1 through B6 contain DNA obtained from cell lines transfected with plasmid pTR-UF5.

Figure 3 shows expression of *lac*Z in recombinant AmEPV-infected mammalian cells. CV-1 cells were mock infected (A) or infected with various AmEPV *lac*Z recombinants, where *lac*Z was under the control of the cowpox virus late ATI gene promoter (B), the late AmEPV spheroidin promoter (C), the *M. melonontha* early *fus* promoter (D) or the AmEPV early *esp* promoter (E). Infection of human Huh-7 liver cells with the AmEPV TK*esp*-lacZ recombinant is also shown as an additional control (F). The infected cell monolayers were stained with X-gal 24h postinfection.

Figure 4 shows the survival of mammalian cells following infection by recombinant AmEPV TK*esp-gfp*. Subconfluent CV-1 cells were infected with AmEPV TK*esp-gfp* at an m.o.i. of 1 PFU/cell. The individual fluorescent cells were located and followed over a period of two to three days and periodically photographed with a fluorescent microscope. One fluorescent cell, identified 18 hours post infection (A), had divided into two cells by 24 (B) to 26 (C) h postinfection and by 50 h had become a small cluster of dividing cells (D).

Figure 5 shows AmEPV-mediated β-galactosidase expression in the muscle of mouse. $2X10^6$ PFU (100 μl) of recombinant AmEPV-*esplacZ* was injected into the muscle of the hind leg of a mouse. As a control, mice were injected with the same amount of recombinant AmEPV-*SPHlacZ* or 100 μl of PBS. Two days later, the mice were sacrificed, the muscle was excised from the injected area and cut into small pieces, and fixed with 3% formaldehyde for 30 min, then stained with X-gal. The muscle injected with recombinant AmEPV pTK-*esplacZ* showed β-galactosidase expression. No β-galactosidase expression was observed in control mice.

Figure 6 shows transformed 293 cells (A) derived from the colony infected with recombinant AmEPV-TKUF5 which are G418 resistant showing that cells are GFP positive, as well as non-fluorescent, non-transformed 293 cells (B).

Figure 7 shows a linear map of the AmEPV genome, 0-139440(A) and 139441-232392(B). Predicted ORFs are numbered consecutively from left to right based upon the position of the initiating methionine codon. ORFs transcribed in a rightward direction are shown above the horizontal line designating the viral genome; ORFs transcribed to the left are below. ITRs are indicated by heavy black arrows. A distance of 1kb is as shown. ChPV homologs are indicated with red numbers, additional MsEPV homologs are indicated with purple numbers. Some ORFs have been assigned function based upon BLAST data.

Figure 8 shows a comparison of the genomic organization of AmEPV, MsEPV and VV. AmEPV ITRs are positioned at the termini of the viral genome as indicated. AmEPV genes which have homology to VV genes are depicted in (A). AmEPV genes which have homology to MsEPV are depicted in (B). Genes in the AmEPV genome common to both MsEPV and VV are in (C). Unique genes encoded by AmEPV are shown in (D).

Figure 9 shows a comparison of the spatial distribution of homologous genes between AmEPV, MsEPV and VV. A random sampling of genes conserved within the genomes of all three indicated viruses were plotted on the 119kb genome of VV, the 232kb AmEPV genome, and the 236kb MsEPV genome. From left to right on the AmEPV genome, the genes shown and their BLAST-assigned function are: AMV016, thymidine kinase; AMV035, membrane protein; AMV038, PAP large subunit; AMV050, DNA polymerase; AMV051, RP035; AMV066, RP0132; AMV105, VETF-L; AMV122, rifampicin resistance; AMV138, no BLAST-assigned function; AMV150, ATP/GTP binding protein; AMV166, RPO19; AMV181, core protein; AMV186, no BLAST-assigned function; AMV205, VLTF-3; AMV221, RPO147; AMV232, membrane protein; AMV243, membrane protein; AMV249, no BLAST-assigned function. Plots compare both orientations of the AmEPV genome. (A) left to right, (B) right to left.

Figure 10 shows residues shared between poxvirus poly(a) polymerase subunit homologs. Consensus shows the conservation between all five sequences. Insect consensus shows identity among the four EPV ORFs. AmEPV consensus displays identities between the two AmEPV subunits.

Figure 11 shows the transmembrane domains possessed by the AmEPV ABC transporter protein. This graphic was produced by the THAMM program (Sonnhammer, E. L. L., Hejne, G., and Krogh, A. [1998] "A hidden Markov model for predicting transmembrane helices inprotein sequences" Proc. of Sixth Int. Conf. on Intelligent Systems for Molecular Biology (J. Glasgow, T. Littlejohn, F. Major, R. Lathrop, D. Sankoff, and C. Sensen, Eds.), pp. 175-182. AAAI press, Menlo Park, CA.). The regions of the protein indicated by the thirteen bars can be seen to have a probability of 1 as transmembrane domains. Although not shown in this figure, the areas between these domains (residues 432-601 and 1097-1285) contain ABC transporter (ATP binding) motifs (Prosite PS00211).

Figure 12 shows the amino acid sequence of the AmEPV serine protease inhibitor. Amino acid abbreviations are standard. The Kunitz family signature (Prostite PS00280) is shown underlined and italicized from residues 55 to 73.

Brief Description of the Sequences

SEQ ID NO: 1 is the nucleotide sequence of the gene encoding AmEPV triacylglyceride lipase.

SEQ ID NO: 2 is the nucleotide sequence of the gene encoding AmEPV Cu^{++}/Zn^{++} superoxide dismutase (SOD).

SEQ ID NO: 3 is the nucleotide sequence of the gene encoding AmEPV CPD photolyase.

SEQ ID NO: 4 is the nucleotide sequence of the gene encoding AmEPV baculovirus-like inhibitor of apoptosis (IAP).

SEQ ID NO: 5 is the nucleotide sequence of the gene encoding a first AmEPV poly(A) polymerase small subunit.

SEQ ID NO: 6 is the nucleotide sequence of the gene encoding a second AmEPV poly(A) polymerase small subunit.

SEQ ID NO: 7 is the nucleotide sequence of the gene encoding a first AmEPV DNA polymerase.

SEQ ID NO: 8 is the nucleotide sequence of the gene encoding a second AmEPV DNA polymerase.

SEQ ID NO: 9 is the nucleotide sequence of the gene encoding AmEPV ABC transporter-like protein.

SEQ ID NO: 10 is the nucleotide sequence of the gene encoding AmEPV Kunitz-motif inhibitor (KPI).

SEQ ID NO: 11 is the nucleotide sequence of the gene encoding AmEPV poly(A) polymerase large subunit.

SEQ ID NO: 12 is the amino acid sequence for the AmEPV triacylglyceride lipase.

SEQ ID NO: 13 is the amino acid sequence for the AmEPV Cu^{++}/Zn^{++} superoxide dismutase (SOD).

SEQ ID NO: 14 is the amino acid sequence for the AmEPV CPD photolyase.

SEQ ID NO: 15 is the amino acid sequence for the AmEPV baculovirus-like inhibitor of apoptosis (IAP).

SEQ ID NO: 16 is the amino acid sequence for the first AmEPV poly(A) polymerase small subunit.

SEQ ID NO: 17 is the amino acid sequence for the second AmEPV poly(A) polymerase small subunit.

SEQ ID NO: 18 is the amino acid sequence for the first AmEPV DNA polymerase.

SEQ ID NO: 19 is the amino acid sequence for the second AmEPV DNA polymerase.

SEQ ID NO: 20 is the amino acid sequence for the AmEPV ABC transporter-like protein.

SEQ ID NO: 21 is the amino acid sequence for the AmEPV Kunitz-motif inhibitor (KPI).

SEQ ID NO: 22 is the amino acid sequence for the AmEPV poly(A) polymerase large subunit.

SEQ ID NOS: 23-27 is the nucleotide sequence of the AmEPV genome.

Detailed Disclosure of the Invention

The subject invention concerns three aspects of entomopoxviruses (EPVs) as novel recombinant vectors: (1) As a system for the expression of high levels of foreign proteins, (2) for the transient expression of foreign genes in mammalian cells and (3) for the stable transformation of vertebrate cells for the long term expression of foreign proteins. In addition, the subject invention provides the nucleotide sequence of the entire genome of genus B entomopoxvirus from Amsacta moorei (AmEPV). Accordingly, the subject invention also concerns isolated polynucleotides encoding AmEPV proteins.

The subject invention concerns novel recombinant vectors and methods for delivery and expression of heterologous polynucleotides in vertebrate cells. The recombinant vectors of the subject invention provide for stable integration and expression of heterologous DNA in the host cell. Advantageously, the vectors of the invention are adapted for accepting large heterologous polynucleotide inserts which can be delivered in an infected or transformed cell and expressed in a stable fashion. The subject invention can be used to provide gene therapy

for conditions or disorders of vertebrate animals, such as a mammal or human, that is in need of such therapy.

One aspect of the subject invention concerns a recombinant EPV vector which can optionally include heterologous DNA which can be expressed in a cell infected or transformed with the subject vector. Preferably, the EPV vector is derived from AmEPV. The recombinant EPV vectors of the present invention can optionally include inverted terminal repeat (ITR) sequences of a virus, such as, for example, adeno-associated virus, that flank the heterologous DNA insertion site on the vector. Thus, when the heterologous DNA is cloned into the recombinant EPV vector, the heterologous DNA is flanked upstream and downstream by the ITR sequences.

In an exemplified embodiment, the subject vectors comprise heterologous DNA inserted within the vector. The heterologous DNA contained within the recombinant vectors of the invention can include polynucleotide sequences which encode a biologically functional protein. Preferably, the polynucleotides encode proteins which can provide therapeutic replacement or supplement in animals afflicted with disorders which result in the animal expressing abnormal or deficient levels of the protein that are required for normal biological function. Proteins encoded by the heterologous DNA can include, but are not limited to interleukins, cytokines, growth factors, interferons, enzymes, and structural proteins. Proteins encoded by the heterologous DNA can also include proteins that provide a selectable marker for expression, such as antibiotic resistance in eukaryotes.

In a preferred embodiment, heterologous DNA within the subject vectors is operably linked with and under the control of regulatory sequences, such as promoters. The recombinant vectors of the invention preferably comprises a constitutive or regulatable promoter capable of promoting sufficient levels of expression of the heterologous DNA contained in the viral vector in a vertebrate cell. Promoters useful with the subject vectors include, for example, the cytomegalovirus (CMV) promoters and the herpes TK gene promoter. The vectors can also include other regulatory elements such as introns inserted into the polynucleotide sequence of the vector.

The strategy for generation of recombinant viruses is identical to that used for VV virus and takes advantage of the high levels of recombination with transfected plasmids mediated by these viruses. The basic procedure utilizes transfection of AmEPV-infected cells with an appropriately designed shuttle vector. Insertion of foreign genes occurs within a non-essential gene (*e.g.*, spheroidin or TK). Because of the cytoplasmic nature of AmEPV, it is necessary to place all foreign genes under control of an AmEPV (early or late) poxvirus promoter. Recombinants are selected and subjected to three rounds of plaque purification before use.

The subject invention also concerns cells containing recombinant vectors of the present invention. The cells can be, for example, vertebrate cells such as mammalian cells. Preferably, the cells are human cells. Cell lines infected or transformed with the recombinant vectors of the present invention are also within the scope of the invention.

The recombinant vectors of the present invention can be introduced into suitable cells or cell lines by methods known in the art. If the recombinant vectors are packaged in viral particles then cells or cell lines can be infected with the virus containing the recombinant vector. Methods contemplated for introducing recombinant vector into cells or cell lines also include transfection, transduction and injection. For example, vectors can be introduced into cells using liposomes containing the subject recombinant vectors. Recombinant viral particles and vectors of the present invention can be introduced into cells by *in vitro* or *in vivo* means.

Infection of vertebrate cells is non-permissive, in that early but not late AmEPV gene expression occurs (Li, Y., R.L. Hall, R.W. Moyer [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562). Specifically, if a reporter gene, such as *lacZ,* is driven by a late poxvirus promoter, such as either the AmEPV spheroidin or cowpox virus ATI (A-type Inclusion) promoter, no expression of galactosidase is observed. If, however, the *lacZ* is driven instead by either of two early EPV promoters (the *Melolontha melolontha* EPV fusolin gene promoter (Gauthier, L., F. Coussrans, J.C. Veyrunes, M. Bergoin [1995] "The *Melolontha melolontha* entomopoxvirus (MmEPV) fusolin is related to the fusolins of lepidoptera EPVs and to the

37 K baculovirus glycoprotein" *Virology* 208:427-436) or the 42 kDa early AmEPV protein (Li *et al.* [1997] *supra*), high levels of galactosidase in the recombinant AmEPV infected vertebrate cells are observed. These results provide clear evidence of AmEPV entry into vertebrate cells followed by early, but not late, viral gene expression.

It has also been found that vertebrate cells survive infection by AmEPV. If CV-1 cells are infected with an AmEPV recombinant which contains the green fluorescent protein (*GFP*) gene regulated by the 42 kDa AmEPV early promoter (also called the *esp* promoter), single, fluorescent cells are initially observed which then proceed to grow and divide, ultimately forming small clusters of fluorescent cells. Therefore, AmEPV enters vertebrate cells, to produce a non-permissive, abortive infection, early viral genes are expressed and infected cells appear to survive and continue to divide. These properties plus a very large capacity of the virus for foreign genes make AmEPV an excellent vector for delivery of genes for expression in a transient fashion.

However, in addition to the ability to express foreign genes in a transient fashion, it has been found that AmEPV vectors of the subject invention have the ability to stably transform cells and express genes in a long term fashion as well. The data presented within the Examples (*e.g.*, Example 2) and accompanying Figures (*e.g.*, Figure 2) confirm that the AmEPV vectors of the subject invention can be used to deliver DNA which subsequently integrates into DNA of the mammalian cell nucleus. The ability of AmEPV to deliver DNA to mammalian cells creates endless opportunity for use of the vector in the stable transformation and engineering of vertebrate cells.

The Examples describe methodology for growth, titration and preparation of recombinant AmEPV, as well as transient expression of AmEPV in vertebrate cells, the use of AmEPV to stably transform mammalian cells, and potential uses of AmEPV vectors.

In addition to entomopoxviruses (EPVs) as novel recombinant vectors, and methods of their use, the subject invention provides the nucleotide sequence of the entire genome of the genus B entomopoxvirus from Amsacta moorei (AmEPV). This enhances the value of AmEPV as a vector, and particularly as a gene therapy vector, in a number of ways. For

example, specific knowledge of the AmEPV genome facilitates the identification of additional sites which may be used as insertion sites for foreign genes of interest.

In addition, the subject invention concerns novel AmEVP polypeptides and the polynucleotide sequences which encode these polypeptides. The AmEPV polynucleotide sequences of the subject invention include polynucleotides encoding a triacylglyceride lipase (SEQ ID NO: 1), a Cu^{++}/Zn^{++} superoxide dismutase (SOD) (SEQ ID NO: 2), a CPD photolyase (SEQ ID NO: 3), a baculovirus-like inhibitor of apoptosis (IAP) (SEQ ID NO: 4), two poly(A) polymerase small subunits (SEQ ID NOS: 5 and 6), two DNA polymerases (SEQ ID NOS: 7 and 8), an ABC transporter-like protein (SEQ ID NO: 9), a Kunitz-motif protease inhibitor (KPI) (SEQ ID NO: 10), and a poly(A) polymerase large subunit (SEQ ID NO: 11) and other polynucleotides.

In addition, the subject invention concerns isolated AmEPV polypeptides encoded by the polynucleotide sequences of the subject invention, including a triacylglyceride lipase (SEQ ID NO: 12), a Cu^{++}/Zn^{++} superoxide dismutase (SOD) (SEQ ID NO: 13), a CPD photolyase (SEQ ID NO: 14), a baculovirus-like inhibitor of apoptosis (IAP) (SEQ ID NO: 15), two poly(A) polymerase small subunits (SEQ ID NOS: 16 and 17), two DNA polymerases (SEQ ID NOS: 18 and 19), an ABC transporter-like protein (SEQ ID NO: 20), a Kunitz-motif protease inhibitor (KPI) (SEQ ID NO: 21), and a poly(A) polymerase large subunit (SEQ ID NO: 22) and other AmEPV polypeptides.

The subject invention includes other AmEPV sequences, as described in Table 1, for example. In addition, the subject invention includes polynucleotides which hybridize with other polynucleotides of the subject invention.

The genome of the genus B entomopoxvirus from *Amsacta moorei* (AmEPV) (SEQ ID NOS: 23-27) was sequenced and found to contain 232,392 bases with 279 unique open reading frames (ORFs) of greater than 60 amino acids. The central core of the viral chromosome is flanked by 9.4kbp inverted terminal repeats (ITRs), each of which contain 13 ORFs, raising the total number of ORFs within the viral chromosome to 292. Default E (EXPECT) values of <0.01 were used to define homology to sequences in current databases. ORFs lacking homology to other poxvirus genes were shown to comprise 33.6% of the viral

genome. Approximately 28.6% of the AmEPV genome (52 AmEPV ORFs) encodes homologues of the mammalian poxvirus co-linear core genes, which are found dispersed throughout the AmEPV chromosome. There is also no significant gene order conservation between AmEPV and the orthopteran genus B poxvirus of *Melanoplus sanguinipes* (MsEPV). Novel AmEPV genes include those encoding an ABC transporter and a Kunitz motif protease inhibitor. The most unusual feature of the AmEPV genome relates to the viral encoded poly(A) polymerase. In all other poxviruses this heterodimeric enzyme consists of a single large and small subunit. However, AmEPV appears to encode one large and two distinct small poly (A) polymerase subunits. AmEPV is one of the few entomopoxviruses which can be grown and manipulated in cell culture.

It is commonly observed in poxvirus genomes that the ORFs situated near the ends of the genome are preferentially transcribed towards the closest termini (Upton, C., Macen, J. L., Maranchuk, R. A., Delange, A. M., and McFadden, G. [1988] "Tumorigenic poxviruses: fine analysis of the recombination junctions in malignant rabbit fibroma virus, a recombinant between Shope fibroma virus and myxoma virus" *Virology* 166, 229-239; Upton, C. and McFadden, G. [1986] "DNA sequence homology between the terminal inverted repeats of Shope fibroma virus and an endogenous cellular plasmid species" *Mol. Cell Biol.* 6 , 265-276). However, as can been seen in Figure 8, this is not the case with the ORFs of AmEPV, where no discernable pattern of transcription of genes near the termini can be observed.

Previous estimates placed the A+T content of the AmEPV genome at 81.5% (Langridge, W. H. R. [1983] "Partial Characterization of DNA from Five Entomopoxviruses" *J. Invertebr.Path* 42, 369-375). A recalculation based upon the known sequence has raised this to 82.2%, making AmEPV the most A+T rich poxvirus sequenced to date. In this regard, it is interesting to note that 62% of all encoded amino acids are either Ile, Leu, Phe, Asn or Lys, in descending order of frequency, which are comprised mainly of A+T rich codons. The unusually high A+T content may also be reflected in the large number of translational stop codons (8.9% of coding capacity) and relatively few methionine encoding triplets (1.6%).

Table 1 lists all the ORFs encoded by the AmEPV genome, and functions assigned to the encoded proteins. Default E (EXPECT) values of <0.01 were used to define homology to sequences in current databases. 52 AmEPV ORFs (28.6% of the genome) show homology to ChPV genes, and 91 ORFs (31.3% of the genome) have homologs in EPVs or other insect viruses. The terminal regions of AmEPV contain few genes homologous to any other gene. Figure 8 illustrates this phenomenon, as well as the observation that AmEPV homologs of both vaccinia and MsEPV genes (which we have used as available examples for the ChPV and EPV) are positioned more towards the centre of the AmEPV genome. In contrast, novel AmEPVgenes are easily identified as occurring more often towards the genomic termini.

Table 1. Predicted ORFs of the AmEPV genome.

ORF	position	aa[a]		Highest blast hit[b]	Expect(E)[c]	aa	Domains[d]	U	E	C[e]	Promoter[f]
AMVITR1	500-1879	460	AF063866	MSV010 Leu rich gene family protein	9.00E-35	611	multiple LRR		x		E
AMVITR2	2108-1929	60					TM	x			E?
AMVITR3	2273-2542	90						x			L
AMVITR4	2934-2545	130					SP	x			E
AMVITR5	3786-2974	271					TM	x			E
AMVITR6	3871-4413	181						x			E
AMVITR7	4872-4600	91						x			E
AMVITR8	6939-5386	518					TM	x			E?,L?
AMVITR9	7018-7221	68					Zinc finger	x			E
AMVITR10	7248-7745	166	P29998	TnGV Enhancin	0.002	901	TM	x			L
AMVITR11	7783-8160	126					TM	x			L?
AMVITR12	8737-8180	186	Z98547	Pf HexExon	4 00E-04	1711	TM	x			E
			X62089	C. botulinum BONT/E	0.001	1251					
AMVITR13	8992-8801	64					TM	x			L?
AMV001	9826-10065	80						x			E
AMV002	10272-10700	143	AF160916	BcDNA LD08534 (D. melan- dUTPase)	2.00E-45	188	dUTPase	x			E?,L?
AMV003	13194-10750	815	AF081810	LdOrf-129 LdNPV	4.00E-39	884	SP, TM	x			E?,L?
AMV004	14087-13278	270						x			E,L
AMV005	15230-14181	350	AF063866	MSV011 Leu rich gene family protein	3 00E-25	505	multiple LRR		x		E,L?
AMV006	15229-15624	132						x			E?,L?
AMV007	15877-15641	79	Z73971	C. elegans MEC-9L protein	7.00E-05	838	Kunitz BPTI, SP	x			E
AMV008	16235-15939	99						x			L?
AMV009	16090-16452	121					TM	x			L?
AMV010	17090-16275	272						x			E
AMV011	17535-17083	151						x			E
AMV012	17288-17545	86					TM	x			
AMV013	18170-17571	200						x			E
AMV014	19693-18236	486	AF063866	MSV240 Leu rich repeat	6.00E-22	527			x		E

ORF	position	aa[a]		Highest blast hit[b]	Expect(E)[c]	aa	Domains[d]	U	E	C[e]	Promoter[f]
AMV015	18363-18614	84					TM	x			L?
AMV016	19763-20308	182	Q05880	CfEPV Thymidine Kinase (J2R)	5.00E-64	185	TK		x	x	E
AMV017	20327-20524	66	P28853	hyp region in Q1 ORF-frameshift?	1.00E-06	66	Leu zipper	x			L
AMV018	20836-20525	104						x			E
AMV019	22494-20923	524	AF162221	XcGV ORF67	6.00E-73	568		x			E
AMV020	22555-23754	400						x			L
AMV021	24548-23757	264	P41436	CpGV IAP	5.00E-80	275	2 BIR	x			E
AMV022	25187-24612	192						x			L?
AMV023	25296-25700	135						x			E
AMV024	25919-26980	354	AF017791	HaEPV 17K orf	3.00E-28	148			x		E?,L?
			AF022176	HaEPV orf6	2.00E-10	286					
AMV025	26995-28353	453	AF063866	MSV235 CPD photolyase	6.00E-91	466	photolyase		x	x	L?
AMV026	28719-28369	117						x			E
AMV027	29077-28775	101						x			E
AMV028	29608-29144	155						x			E
AMV029	30545-29676	290	AF063866	MSV027 Trp repeat gene family protein	4.00E-50	297			x		E
AMV030	31173-30742	144						x			E
AMV031	31640-32092	151					TM	x			E
AMV032	32570-32100	157	U30297	{AmEPV FALPE}				x			L
AMV033	32689-33975	429	AF063866	MSV019 hp	3.00E-25	437			x		L
AMV034	34365-33976	130	AF019224	HaEPV F4L	4.00E-08	85			x		E
AMV035	34428-35435	336	AF063866	MSV121 membrane protein (G9R)	3.00E-86	333			x	x	L?
AMV036	35313-35104	70						x			L?
AMV037	36182-35442	247	X95275	Pf frameshift	2.00E-04	960		x			L
AMV038	37923-36205	573	AF063866	MSV143 PAP-L (E1L)	1.00E-125	571			x	x	L?
AMV039	38018-39613	532						x			L?
AMV040	40159-39608	184	AF063866	MSV138 hp	0.002	190			x		L
AMV041	40203-40841	213	AF063866	MSV039 (G6R)	1.00E-43	193			x	x	L
AMV042	40858-41205	116						x			L?
AMV043	41228-41428	67	AF063866	MSV188 hp	3.00E-07	68			x		E,L
AMV044	43176-41431	582	AF063866	MSV140 hp	3.00E-17	608	TM		x		L
AMV045	45167-43206	654	AF063866	MSV077 hp	1.00E-15	598	TM		x		E
AMV046	43777-43962	62					TM	x			E?
AMV047	45255-46031	259	AF063866	MSV187 VLTF-2 (A1L)	5.00E-41	261			x	x	L
AMV048	47092-46034	353	AF063866	MSV156 hp	8.00E-06	1127			x		L?
AMV049	47212-47772	187						x			L?
AMV050	51077-47763	1105	X57314	CbEPV DNA polymerase (E9L) [36]	*	964	DNA pol B		x	x	E
AMV051	52177-51131	349	AF063866	MSV149 RPO35 (A29L)	8.00E-54	348			x	x	E
AMV052	52298-53296	333	AF063866	MSV130 DNA topoisomerase (H6R)	1.00E-106	328	TM		x	x	L
AMV053	54234-53299	312	AF063866	MSV120 hp	4.00E-10	251			x		E
AMV054	54298-56763	822	AF063866	MSV119 RAP94 (H4L)	1.00E-150	807	TM		x	x	L
AMV055	57258-56860	133	AF022176	HaEPV orf6	2.00E-14	286			x		E
			AF063866	MSV194 ALI motif	1.00E-10	409					
AMV056	57332-57589	86					TM	x			
AMV057	58350-57292	353	AF022176	HaEPV orf6	4.00E-90	286			x		E
			AF063866	MSV194 ALI motif	8.00E-14	409					
AMV058	58496-59323	276	AF063866	MSV150 NTP pyrophosphohydorlase / mutT (D10R)	1.00E-18	289	MutT		x	x	E
AMV059	59361-60761	469	AF063866	MSV148 DNA helicase (A18R)	5.00E-78	471	DEAD box/ helicase C		x	x	L?
AMV060	60806-61690	295	AF022176	HaEPV PAP reg subunit (J3R) [41]	1.00E-102	293	PARP reg		x	x	E?,L?
AMV061	62470-61706	255	AF022176	HaEPV 30K virion protein (L4R) [158]	4.00E-92	293			x	x	L
AMV062	62518-63009	164	AF022176	HaEPV orf4 [160]	1.00E-61	166			x		E?,L?
AMV063	63072-63686	205						x			L?

ORF	position	aa[a]	Highest blast hit[b]		Expect(E)[c]	aa	Domains[d]	U	E	C[e]	Promoter[f]
AMV064	64223-63696	176						x			E
AMV065	63919-64113	65					TM	x			L?
AMV066	64284-67871	1196	AF063866	MSV155 RNApol RPO132 (A24R)	*	1190			x	x	L
AMV067	65029-64847	61						x			
AMV068	68446-67892	185					TM	x			E
AMV069	69548-68505	348	AF063866	MSV180 (L3L)	8.00E-59	343			x	x	L
AMV070	69602-70357	252						x			E
AMV071	70684-70358	109	AF063866	MSV049 hp	2.00E-17	116	TM		x		L?
AMV072	70698-71168	157	AF063866	MSV044 hp	3 00E-20	165			x		L
AMV073	71234-71485	84						x			L?
AMV074	71866-71549	106					SP, TM	x			L?
AMV075	71613-72086	158						x			E
AMV076	72586-72236	117	AF063866	MSV255 Leu rich gene family protein	1 00E-09	403			x		L?
AMV077	72369-72629	87					TM	x			
AMV078	73085-73579	165	AF108960	SeNPV protein-Tyr phosphatase	6.00E-25	165	DSPc	x		x	L
AMV079	73874-73668	69	AF063866	MSV087 Thioredoxin	1 00E-08	76			x		L
AMV080	74247-73870	126	AF063866	MSV085 hp	1.00E-04	118	TM, Leu zipper		x		E,L
AMV081	76410-74251	720	AF063866	MSV086 RNA helicase (I8R)	1.00E-172	717	DEAD box/ helicase C		x	x	L?
AMV082	76620-76435	62						x			L?
AMV083	76627-77028	134					TM	x			L
AMV084	77056-77865	270	U87984	D. melan. ovarian spec. Ser/Thr kinase	2.00E-13	459	2 pkinase	x			E
AMV085	77906-78517	204	AF063866	MSV088 hp	2.00E-14	205			x		L
AMV086	79422-79138	95						x			E
AMV087	81627-79450	726	AF063866	MSV089 NTPase (D5R)	1 00E-139	834			x	x	E
AMV088	81771-82097	109						x			L
AMV089	82126-82437	104					TM	x			L
AMV090	83228-82440	263	AF063866	MSV116 hp	3 00E-08	317			x		E
AMV091	84321-83254	356	AF063866	MSV052 (A23R)	3.00E-38	345			x	x	E
AMV092	83537-83842	102					TM	x			L?
AMV093	85132-84347	262	AF063866	MSV124 mRNA capping small subunit (D12L)	3.00E-53	267			x	x	E?,L
AMV094	86177-85998	60							x		L?
AMV095	86119-86310	64					TM	x			
AMV096	87404-86394	337	AF063866	MSV213 hp	6.00E-86	331			x		L
AMV097	87220-87405	62						x			E
AMV098	87478-87903	142	AF063866	MSV136 hp	5 00E-12	150			x		L
AMV099	89237-87918	440	AF063866	MSV071 hp	7.00E-32	442			x		L?
AMV100	89670-89263	136	AF017791	HaEPV 17K ORF	5.00E-05	148			x		E,L
AMV101	90120-89695	142	AF063866	MSV079 hp with C2H2 zinc finger	1 00E-19	138			x		E,L
AMV102	90585-90142	148	AF063866	MSV092 hp	2.00E-12	196			x		E?,L?
AMV103	90272-90475	68						x			L?
AMV104	91030-90572	153						x			E
AMV105	91081-93381	767	AF063866	MSV063 VETF-L (A7L)	*	760			x	x	E,L
AMV106	93290-92931	120					TM	x			E?,L?
AMV107	93837-93391	149						x			E
AMV108	93494-93736	81					TM	x			E?,L?
AMV109	93941-95290	450	AF162221	XcGV ORF22	6 00E-76	492		x			E
AMV110	95332-96417	362	AF017791	HaEPV 17K ORF	5.00E-28	148			x		E
AMV111	95942-95700	81					TM	x			L?
AMV112	96452-97495	348	AF017791	HaEPV 17K ORF	3 00E-30	148			x		E?
AMV113	97020-96820	67					SP, TM	x			L?
AMV114	97527-97841	105	AF063866	MSV093 put. redox (E10R)	2 00E-27	107	SP		x	x	L

ORF	position	aa[a]		Highest blast hit[b]	Expect(E)[c]	aa	Domains[d]	U	E	C[e]	Promoter[f]
AMV115	97853-98731	293	AF063866	MSV041 PAP-S (J3R)	1.00E-27	295	PARP reg		x	x	L?
AMV116	99126-98734	131						x			L?
AMV117	99484-99131	118						x			L?
AMV118	100672-99515	386	AF063866	MSV090 put membrane protein (A16L)	1.00E-121	380	TM		x	x	L
AMV119	102089-101016	358	AF063866	MSV081 PP2C	4.00E-69	357	PP2C		x		L
AMV120	102151-102570	140	AF063866	MSV082 hp	2.00E-08	139			x		L
AMV121	103396-102581	272	AF063866	MSV064 hp	2.00E-31	280			x		E?,L?
AMV122	105388-103688	567	U44841	HaEPV rifampicin resistance gene (D13L) [69]	*	584			x	x	E?
AMV123	105901-105470	144					TM	x			E
AMV124	107828-105948	627					TM	x			L
AMV125	107560-107739	60					TM	x			L?
AMV126	108199-107915	95						x			E
AMV127	109346-108762	195	AF063866	MSV060 (H2R)	1.00E-57	194	TM		x	x	L
AMV128	110119-109364	252					TM	x			E
AMV129	110338-110156	61						x			E?,L?
AMV130	110459-114610	1384	Z82272	C elegans- similar to ABC transporters	5e-54**	1431	TM	x			E
AMV131	115711-114941	257						x			E
AMV132	116352-115732	207	AF017791	HaEPV 17K ORF	0.001	148			x		E
AMV133	117243-116383	287	AF063866	MSV048 lipase	1.00E-56	288	lipase 3, TM		x	x	L
AMV134	118889-117285	535	AF063866	MSV240 Leu rich repeat {AmEPV Q3 }	5.00E-15	527			x		E
AMV135	121563-118948	872	AF063866	MSV067 put mRNA capping large subunit (D1R)	*	860			x	x	E
AMV136	120638-120928	97					TM	x			E
AMV137	121578-122222	215	AF063866	MSV068 hp	2 00E-15	160	TM		x		L
AMV138	123184-122225	320	AF063866	MSV151 (A11R)	5.00E-29	313			x	x	L
AMV139	123209-126655	1149	AF063866	MSV152 P4a (A10L)	1e-63/6e-29	1306			x	x	L
AMV140	127596-126667	310	AF063866	MSV170 hp	3.00E-07	324			x		E
AMV141	127730-129085	452	AF063866	MSV050 hp	5.00E-57	379	TM		x		L?
AMV142	128757-128554	68					TM	x			
AMV143	129503-129061	141					TM	x			L?
AMV144	129837-129493	115					TM	x			E
AMV145	130422-129880	181	AF063866	MSV167 hp	2.00E-15	178			x		E,L
AMV146	129909-130115	69					SP	x			L?
AMV147	130483-132486	668	AF063866	MSV164 core protein (A3L)	1.00E-146	648	TM		x	x	L
AMV148	132955-132489	156					TM	x			E
AMV149	133439-133008	144					TM	x			E
AMV150	134239-133520	240	AF063866	MSV171 ATP/GTP binding protein (A23L)	1.00E-43	244			x	x	L?
AMV151	134280-134930	217	AF063866	MSV172 hp	0 77	184			x		L
AMV152	134554-134778	75					TM	x			E
AMV153	134987-136390	468	AF063866	MSV173 Ser/Thr protein kinase (F10L)	4.00E-73	457	TM		x	x	E,L
AMV154	135283-135086	66						x			
AMV155	136164-135970	65					TM	x			L?
AMV156	140090-136377	1238	AF063866	MSV156 hp	3.00E-28	1127			x		E?,L?
AMV157	140145-140876	244	AF063866	MSV169 hp	9.00E-12	230	TM		x		L?
AMV158	140599-140254	116					TM	x			L?
AMV159	141543-140890	218	AF063866	MSV111 hp	0 001	201	TM		x		L?
AMV160	142175-141549	209	AF063866	MSV110 hp	0 16	181	TM		x		E
AMV161	142449-142207	81	AF063866	MSV108 hp	1 00E-11	76	TM		x		L
AMV162	142949-142461	163	AF063866	MSV106 (A22R)	3.00E-26	163			x	x	L?

ORF	position	aa[a]		Highest blast hit[b]	Expect(E)[c]	aa	Domains[d]	U	E	C[e]	Promoter[f]
AMV163	143230-142955	92	AF063866	MSV112 hp	0.042	130	TM		x		E
AMV164	143963-143256	236	AF063866	MSV107 hp	1.00E-26	226	TM		x		L
AMV165	145086-144112	325					TM	x			E
AMV166	145849-145139	237	AF063866	MSV100 RPO19 (A5R)	1.00E-35	230			x	x	E,L
AMV167	146277-146035	81	U16956	F. neoformas polyubiquitin [144]	1.00E-34	381	ubiquitin		x		E
AMV168	146669-146316	118	AF063866	MSV165 hp	1.00E-04	126	TM		x		E?,L
AMV169	146862-147086	75					TM	x			L
AMV170	147105-148697	531	AF063866	MSV145 hp	3.00E-83	525			x		L
AMV171	148735-149010	92	AF063866	MSV166 hp	4.00E-24	96	TM		x		L
AMV172	149358-149017	114	AF063866	MSV098 hp	6.00E-04	108			x		L
AMV173	149405-150724	440	AF063866	MSV157 hp	9.00E-20	430			x		E
AMV174	152725-150716	670	AF063866	MSV113 VETF-s (D6R)	*	674	SNF2N/ helicase C, TM		x	x	E
AMV175	153799-152762	346	AF022176	HaEPV orf6	2 00E-75	286			x		E
			AF063866	MSV194 ALI motif	3 00E-14	409					
AMV176	152802-153059	86					TM, SP	x			
AMV177	154912-153833	360	AF022176	HaEPV orf6	6.00E-73	286			x		E
			AF063866	MSV194 ALI motif	6.00E-11	409					
AMV178	153873-154130	86					TM, SP	x			
AMV179	154996-156243	416	AF063866	MSV115 (G5R)	3.00E-29	505			x	x	E
AMV180	156293-156784	164						x			E
AMV181	158275-156884	464	Af063866	MSV189 core protein [G1L] (I7L)	1.00E-112	443			x	x	L?
AMV182	157358-157552	65						x			L?
AMV183	158290-158964	225	AF063866	MSV190 {AmEPV G2R}	5.00E-09	227	SP		x		L
AMV184	158990-158745	82					TM	x			L?
AMV185	159291-159058	78	M77182	{AmEPV G3L}	2.00E-35	78		x			E
AMV186	159318-159800	161	AF063866	MSV132 (A28L)	2 00E-45	142	TM		x	x	L?
AMV187	159896-162904	1003	U19239	CfEPV spheroidin [76]	*	999	TM, Leu zipper		x		L
AMV188	161562-161383	60					TM	x			
AMV189	162575-162396	60						x			
AMV190	162767-162585	61						x			L?
AMV191	162621-162848	76					TM	x			E?
AMV192	165039-163096	648	AF027657	CfEPV NPH-I (D11L) [53]	*	647	SNF2N/ helicase C		x	x	L
AMV193	165514-165065	150	U83981	H. sapiens apoptosis-associated protein	4.00E-04	674		x			L?
AMV194	165666-167081	472	AF063866	MSV198 MTG motif gene family protein	7.00E-46	399			x		E
AMV195	169144-167255	630	AE001415	Pf hp	1.00E-04	1351	TM	x			L
AMV196	168955-169134	60					TM	x			L?
AMV197	169246-170142	299	AF170726	Myxoma virus m142R (B1R) [154]	1.00E-27	306	2 pkinase		x	x	E
AMV198	171035-170724	104	AF019224	HaEPV ORF F2L [161]	1 00E-21	101	TM		x		L
AMV199	171052-172647	532	AF063866	MSV162 NAD+dep DNA ligase	1.00E-100	522	DNA ligase N		x		L?
AMV200	172798-173481	228	AF063866	MSV159 hp	4.00E-18	225			x		L
AMV201	173525-173881	119					TM	x			E
AMV202	173835-173617	73					TM	x			L?
AMV203	174115-173888	76	AF063866	MSV168 hp	5.00E-06	72			x		L
AMV204	174147-174395	83					TM	x			L
AMV205	175077-174394	228	AF063866	MSV065 VLTF-3 (A2L)	1 00E-47	218			x	x	L
AMV206	175140-175601	154						x			E
AMV207	177028-175601	476	AF063866	MSV198 MTG motif gene family protein	6.00E-87	399	TM		x		L?
AMV208	176467-176670	68					TM	x			
AMV209	178433-177045	463	AF063866	MSV198 MTG motif gene family protein	5.00E-97	399	TM		x		E?

ORF	position	aa[a]		Highest blast hit[b]	Expect(E)[c]	aa	Domains[d]	U	E	C[e]	Promoter[f]
AMV210	180326-178491	612	AF063866	MSV117 DNA pol beta/AP polymerase	1.00E-122	603	AP endo 2/ DNA pol X		x		L?
AMV211	180741-180322	140	AF063866	MSV137 hp	3.00E-19	149			x		L
AMV212	181674-180823	284	M24328	Pf Asp-rich protein [261]	1.00E-05	537			x		E
AMV213	181926-181720	69						x			E
AMV214	183172-181961	404	AF063866	MSV184 hp	8.00E-54	415	TM		x		E
AMV215	182265-182546	94					TM	x			L?
AMV216	184838-183216	541	AF063866	MSV099 hp	1.00E-41	519	TM		x		E
AMV217	184913-185650	246	AF063866	MSV183 myristylated membrane protein (L1R)	5.00E-72	242	TM		x	x	L?
AMV218	185690-185968	93	L27838	P. yoelii rhoptry protein	0.006	2269		x			E
AMV219	186862-185966	299	AF063866	MSV072 hp	1.00E-45	298			x		L
AMV220	187176-186958	73					TM	x			
AMV221	187007-190909	1301	AF063866	MSV043 RPO147 (J6R)	*	1319	RNA pol A		x	x	E
AMV222	190095-189904	64					TM	x			L?
AMV223	190945-191358	138					TM	x			E
AMV224	192158-191589	190	AE001145	B burgdorferi pred. coding region BB0398	0 001	343		x			L?
AMV225	192699-193253	185						x			L?
AMV226	193743-193252	164	AF063866	MSV031 hp	7 00e-15**	141			x		L
AMV227	193457-193714	86					TM	x			L?
AMV228	194218-193739	160	AF063866	MSV097 put. Ca2+ BP	5 00E-15	140	2 EFhand		x		E?
AMV229	194453-194229	75						x			E?
AMV230	194544-195080	179	AF063866	MSV245 RPO18 (D7R)	3 00E-20	186			x	x	E,L
AMV231	195253-195984	244	AF063866	MSV208 uracil DNA glycosylase (D4R)	3.00E-38	232	2 UNG		x	x	E
AMV232	196415-195996	140	AF063866	MSV142 put membrane protein (J5L)	3 00E-36	139	TM		x	x	L
AMV233	196480-197103	208	AF063866	MSV032 hp	4.00E-20	252			x		L
AMV234	197134-197844	237	AF063866	MSV135 put. protein phosp hatase 2C	3.00E-27	239	PP2C		x		E?,L
AMV235	197847-198521	225	AF063866	MSV123 hp	1.00E-24	230			x		L?
AMV236	199146-198517	210						x			E
AMV237	199209-199445	79						x			L
AMV238	199452-200795	448	AF063866	MSV055 hp	7.00E-09	466	TM		x		E,L
AMV239	200248-199973	92					TM	x			L?
AMV240	201591-200794	266	U42580	Paramecium bursaria chlorella virus A467L	8.00E-07	312		x			E?,L
AMV241	201853-201638	72						x			L
AMV242	201954-202283	110						x			E
AMV243	203059-202316	248	AF063866	MSV094 put membrane protein (F9L)	7 00E-35	241	TM		x	x	L?
AMV244	202716-203075	120					TM	x			
AMV245	203101-203577	159						x			E
AMV246	204042-203572	157	L33180	AcNPV phosphotyrosine phosphatas e	5.00E-28	168	DSPc	x			E
AMV247	204194-204610	139	AF063866	MSV139 hp	1.00E-07	139			x		L
AMV248	204830-205696	289	AF063866	MSV206 put glycosyltransferase	1.00E-42	287	TM		x		L?
AMV249	205711-206046	112	AF063866	MSV209 (A21L)	2.00E-24	113	TM		x	x	L
AMV250	206114-206419	102						x			E
AMV251	206367-206155	71					SP	x			E
AMV252	206716-206474	81					TM	x			L?
AMV253	206768-208222	485	X95275	Pf frameshift	6.00E-04	960		x			E
AMV254	208261-208905	215	AF063866	MSV027 Trp gene family protein	1.00E-19	297			x		E
AMV255	208973-209428	152	P24705	AcNPV superoxide dismutase	1 00E-47	151	SODCu	x		x	L?
AMV256	211257-209431	609	AF063866	MSV056 metalloprotease (G1L)	4.00E-05	629	TM		x	x	L
AMV257	211349-211723	125	AF003534	Chilo iridescent virus O11L [196]	2 00E-14	230			x		L?

ORF	position	aa[a]		Highest blast hit[b]	Expect(E)	aa	Domains[d]	U	E	C[e]	Promoter[f]
AMV258	211785-214262	826	X95275	Pf frameshift	9 00E-04	960	TM	x			E?
AMV259	214913-214488	142						x			E
AMV260	216480-214969	504	X95275	Pf frameshift	3.00E-12	960		x			E
AMV261	216586-217788	401					TM	x			E
AMV262	218411-217797	205						x			E?,L?
AMV263	219301-218438	288	AF067136	PP1 reg subunit 7 hSDS22 homolog [261]	8.00E-19	360	multiple LRR		x		E?
AMV264	220213-219377	279	AF063866	MSV099 hp	7.00E-04	519	TM		x		E
AMV265	221229-220318	304					TM	x			E
AMV266	221858-221307	184						x			E

[a] amino acids, [b] GenBank accession numbers, [c] likelihood of identity (EXPECT) score, [d] predicted domains revealed by Pfam and Psort programs (see materials and methods), [e] U = genes not found in other poxviruses; E = genes found in other entomopoxviruses; C = genes found in chordopoxviruses, [f] promoter type E = early; L = late, promoters with ambiguous motifs designated by ?. hp = hypothetical protein. * designates an E score too low to be quantified. Vaccinia homologs are shown in parentheses, MsEPV homologs not already listed are in brackets, braces contain formerly named AmEPV genes.

Vertebrate poxviruses have been shown to generally share a co-linear arrangement of core genes (Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. [1990] "The complete DNA sequence of vaccinia virus" *Virology* 179, 247-66, 517-63; Massung, R. F., Liu, L. I., Qi, J., Knight, J. C., Yuran, T. E., Kerlavage, A. R., Parsons, J. M., Venter, J. C., and Esposito, J. J. [1994] "Analysis of the complete genome of smallpox variola major virus strain Bangladesh-1975" *Virology* 201:215-240; Senkevich, T. G., Koonin, E. V., Bugert, J. J., Darai, G., and Moss, B. [1997] "The genome of molluscum contagiosum virus: Analysis and comparison with other poxviruses" *Virology* 233:19-42; Afonso, C. L., Tulman, E. R., Lu, Z., Zsak, L., Kutish, G. F., and Rock, D. L. [2000] "The Genome of Fowlpox Virus" *J. Virol* 74:3815-3831). Sequence information from a number of EPVs suggested that this co-linear arrangement of core genes is not conserved in members of the EPV subfamily (Hall, R. L. and Moyer, R. W. [1993] "Identification of an *Amsacta* spheroidin-like protein within the occlusion bodies of *Choristoneura* entomopoxviruses" *Virology* 192:179-187; Sriskantha, A., Osborne, R. J., and Dall, D. J. [1997] "Mapping of the Heliothis armigera entomopoxvirus (HaEPV) genome, and analysis of genes encoding the HaEPV spheroidin and nucleoside triphosphate phosphohydrolase I proteins" *J Gen Virol* 78:3115-3123; Afonso, C. L., Tulman, E. R., Lu, Z., Oma, E., Kutish, G. F., and Rock, D. L. [1999] "The genome of Melanoplus sanguinipes Entomopoxvirus" *J. Virol.* 73, 533-552). The complete genomic sequence of AmEPV enables us to unequivocally confirm this, but also shows there is no conserved co-linear core

between viruses of genus B. Figure 9 graphically illustrates the absence of any type of shared spatial gene arrangement between a typical ChPV (VV), MsEPV and the genome of AmEPV. Note that flipping the AmEPV genome direction from 3' to 5' does not lessen the degree of gene shuffling which has occurred within these different viruses.

Promoter consensus sequences

AmEPV contains promoter elements which govern gene expression. 133 AmEPV genes are considered to be early, or potentially early. 158 genes possess motifs which result in late, or potentially late promoters. Only 15 genes from the entire 279 gene genome have no recognizable promoter or regulatory elements. Genes that contain the sequences TGAAAXXXXA or TGAATXXXXA within 100 bases of their translational start codons were considered early (E) or potentially early (E?), respectively (Table 1). This motif resembles the ChPV early promoter core consensus sequence (Moss, B. [1996] *Poxviridae*: The viruses and their replication. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 2637-2672, Lippincott-Reven, Philadelphia; Senkevich, T. G., Koonin, E. V., Bugert, J. J., Darai, G., and Moss, B. [1997] "The genome of molluscum contagiosum virus: Analysis and comparison with other poxviruses" *Virology* 233:19-42) and was also used to predict early genes of MsEPV (Afonso, C.L., E.R. Tulman, Z. Lu, E. Oma, G.F. Kutish, and D.L. Rock [1999] "The genome of *Melanoplus sanguinipes* Entomopoxvirus" J. Virol. 73:533-552). These motifs have been found upsteam of known EPV early genes such as the TK gene (Gruidl, M. E., Hall, R. L., and Moyer, R. W. [1992] "Mapping and molecular characterization of a functional thymidine kinase from *Amsacta moorei* entomopoxvirus" *Virology* 186:507-516; Lytvyn, V., Fortin, Y., Banville, M., Arif, B., and Richardson, C. [1992] "Comparison of the thymidine kinase genes from three entomopoxviruses" *J. Gen. Virol.* 73:3235-3240), CbEPV DNA polymerase (Mustafa, A. and Yuen, L. [1991] "Identification and sequencing of the Choristoneura biennis entomopoxvirus DNA polymerase gene" *DNA Seq.* 2:39-45), and the MmEPV fusolin gene (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562; Gauthier, L.,

Cousserans, F., Veyrunes, J. C., and Bergoin, M. [1995] "The Melolontha melolontha entomopoxvirus (MmEPV) fusolin is related to the fusolins of lepidopteran EPVs and to the 37K baculovirus glycoprotein" *Virology* 208:427-436). Out of the 36 early MsEPV gene homologs in AmEPV, 27 contain predicted early promoters. For comparison with vertebrate poxvirus homologs, we adopted the most recently published mammalian poxvirus promoter sequence predictions (Cameron, C., Hota-Mitchell, S., Chen, L., Barrett, J., Cao, J. X., Macaulay, C., Willer, D., Evans, D., and McFadden, G. [1999] "The complete DNA sequence of myxoma virus" *Virology* 264:298-318; Willer, D. O., McFadden, G., and Evans, D. H. [1999] "The complete genome sequence of shope (Rabbit) fibroma virus" *Virology* 264:319-343), which emanated from the earlier work of Davison and Moss (Davison, A. J. and Moss, B. [1989] "Structure of vaccinia virus late promoters" *J. Mol. Biol.* 210:771-784). Out of the 16 early MYX homologs in AmEPV, 11 contained early promoter elements. As a first approximation, candidate genes were considered early only if they contained the vaccinia virus early transcription termination sequence (TTTTTNT) near the 3' end of the gene; (Gruidl, M. E., Hall, R. L., and Moyer, R. W. [1992] "Mapping and molecular characterization of a functional thymidine kinase from *Amsacta moorei* entomopoxvirus" *Virology* 186:507-516; Li, X., Barrett, J. W., Yuen, L., and Arif, B. M. [1997] "Cloning, sequencing and transcriptional analysis of the Choristoneura fumiferana entomopoxvirus spheroidin gene" *Virus Res.* 47:143-154; Sriskantha, A., Osborne, R. J., and Dall, D. J. [1997] "Mapping of the Heliothis armigera entomopoxvirus (HaEPV) genome, and analysis of genes encoding the HaEPV spheroidin and nucleoside triphosphate phosphohydrolase I proteins" *J Gen Virol* 78:3115-3123; Yuen, L. and Moss, B. [1987] "Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes" *Proc. Natl. Acad. Sci. U.S.A.* 84:6417-6421). There are examples of early genes which contain the TTTTTNT motif towards the 5' N-terminal portion of the ORF such as the DNA polymerase of myxoma (Cameron, C., Hota-Mitchell, S., Chen, L., Barrett, J., Cao, J. X., Macaulay, C., Willer, D., Evans, D., and McFadden, G. [1999] "The complete DNA sequence of myxoma virus" *Virology* 264:298-318) and Shope fibroma (Willer, D. O., McFadden, G., and Evans, D. H. [1999] "The complete genome sequence of shope (Rabbit) fibroma virus" *Virology*

264:319-343) viruses. Therefore, our estimates of early genes may be low. This sequence is found within 100 bases 3' of the ORF of 42 of the 116 predicted early AmEPV genes.

AmEPV ORFs that contained the sequence TAAATG at the translational start site were considered late genes (L) (Bertholet, C., Stocco, P., Van Meir, E., and Wittek, R. [1986] "Functional analysis of the 5' flanking sequence of a vaccinia virus late gene" *EMBO J.* 5:1951-1957; Rosel, J. and Moss, B. [1985] "Transcriptional and translational mapping and nucleotide sequence analysis of a vaccinia virus gene encoding the precursor of the major core polypeptide 4b" *J. Virol.* 56:830-838; Weir, J. P. and Moss, B. [1984] "Regulation of expression and nucleotide sequence of a late vaccinia virus gene" *J. Virol.* 51:662-669; Davison, A. J. and Moss, B. [1989] "Structure of vaccinia virus late promoters" *J. Mol. Biol.* 210:771-784). This late promoter consensus sequence has been observed in other EPV late genes such as spheroidin, the nucleoside triphosphatase (NTPase), and hydrolase I (NPH-I), and topoisomerase (Hall, R. L., Li, Y., Feller, J. A., and Moyer, R. W. [1996] "The *Amsacta moorei* entomopoxvirus spheroidin gene is improperly transcribed in vertebrate poxviruses" *Virology* 224:427-436; Hall, R. L. and Moyer, R. W. [1991] "Identification, cloning, and sequencing of a fragment of *Amsacta moorei* entomopoxvirus DNA containing the spheroidin gene and three vaccinia virus-related open reading frames" *J. Virol.* 65:6516-6527; Li, X., Barrett, J. W., Yuen, L., and Arif, B. M. [1997] "Cloning, sequencing and transcriptional analysis of the Choristoneura fumiferana entomopoxvirus spheroidin gene" *Virus Res.* 47:143-154; Sanz, P., Veyrunes, J. C., Cousserans, F., and Bergoin, M. [1994] "Cloning and sequencing of the spherulin gene, the occlusion body major polypeptide of the Melolontha melolontha entomopoxvirus (MmEPV)" *Virology* 202:449-457; Sriskantha, A., Osborne, R. J., and Dall, D. J. [1997] "Mapping of the Heliothis armigera entomopoxvirus (HaEPV) genome, and analysis of genes encoding the HaEPV spheroidin and nucleoside triphosphate phosphohydrolase I proteins" *J Gen Virol* 78:3115-3123). Genes that contained the sequences TAAAT or TAAAAT within 100 bases upstream from their start codon were also potentially considered late genes (L?) (Table 1). These sequences have been found upstream of other late vertebrate poxvirus genes (Roseman, N. A. and Hruby, D. E. [1987] "Nucleotide sequence and transcript organization of a region of

the vaccinia virus genome which encodes a constitutively expressed gene required for DNA replication" *J. Virol.* 61:1398-1406).

Terminal regions

AmEPV is one of the few entompoxviruses which can be easily and reliably replicated in tissue culture (Winter, J., Hall, R. L., and Moyer, R. W. [1995] "The effect of inhibitors on the growth of the entomopoxvirus from Amsacta moorei in Lymantria dispar (gypsy moth) cells" *Virology* 211: 462-473; Hall, R. L., Li, Y., Feller, J. A., and Moyer, R. W. [1996] "The *Amsacta moorei* entomopoxvirus spheroidin gene is improperly transcribed in vertebrate poxviruses" *Virology* 224:427-436). Because of this, we were able to obtain DNA for sequencing from a single clonal virus plaque, thus minimizing template heterogeneity. The results of sequencing from non-clonally isolated template DNA can be seen in the resultant sequence of MsEPV, where the two inverted terminal repeat (ITR) regions are not identical (Afonso, C. L., Tulman, E. R., Lu, Z., Oma, E., Kutish, G. F., and Rock, D. L. [1999] "The genome of Melanoplus sanguinipes Entomopoxvirus" *J. Virol.* 73, 533-552).

Poxvirus ITRs can vary considerably in size. The smallest ITRs are those of variola Bangladesh which are only 725 bp (Massung, R. F., Esposito, J. J., Liu, L. I., Qi, J., Utterback, T. R., Knight, J. C., Aubin, L., Yuran, T. E., Parsons, J. M., Loparev, V. N., Selivanov, N. A., Cavallaro, K. F., Kerlavage, A. R., Mahy, B. W. J., and Venter, J. C. [1993] "Potential virulence determinants in terminal regions of variola smallpox virus genome" *Nature* 366:748-751; Massung, R. F., Liu, L. I., Qi, J., Knight, J. C., Yuran, T. E., Kerlavage, A. R., Parsons, J. M., Venter, J. C., and Esposito, J. J. [1994] "Analysis of the complete genome of smallpox variola major virus strain Bangladesh-1975" *Virology* 201:215-240). The AmEPV genome contains identical ITR tandem repeats of 9.4 kbp at both termini which are organized in a fashion similar to that of other poxviruses; i.e. a series of tandemly repeated sequences interspersed with non-repetitive spacer region (Figure 1) (Massung, R. F., Knight, J. C., and Esposito, J. J. [1995] "Topography of variola smallpox virus inverted terminal repeats" *Virology* 211:350-355; Wittek, R., Menna, A., Muller, H.

K., Schumperli, D., Boseley, P. G., and Wyler, R. [1978] "Inverted terminal repeats in rabbit poxvirus and vaccinia virus DNA" *J. Virol.* 28:171-181; Upton, C. and McFadden, G. [1986] "DNA sequence homology between the terminal inverted repeats of Shope fibroma virus and an endogenous cellular plasmid species" *Mol. Cell Biol.* 6 , 265-276; Afonso, C. L., Tulman, E. R., Lu, Z., Oma, E., Kutish, G. F., and Rock, D. L. [1999] "The genome of Melanoplus sanguinipes Entomopoxvirus" *J. Virol.* 73, 533-552). Myxoma virus and AmEPV share a similar ITR structure, in that the ORFs encoded in this region extend to the very ends of the genome termini, and contain very little non-coding DNA (Cameron, C., Hota-Mitchell, S., Chen, L., Barrett, J., Cao, J. X., Macaulay, C., Willer, D., Evans, D., and McFadden, G. [1999] "The complete DNA sequence of myxoma virus" *Virology* 264:298-318). Other sequenced poxvirus ITRs contain smaller numbers of genes interspersed with large regions of non-coding DNA within them. Other examples of poxvirus ITRs include MsEPV, which contains 3kb of non-coding DNA within the ITRs (Afonso, C. L., Tulman, E. R., Lu, Z., Oma, E., Kutish, G. F., and Rock, D. L. [1999] "The genome of Melanoplus sanguinipes Entomopoxvirus" *J. Virol.* 73, 533-552), 6kb in VV Ankara strain (Antoine, G., Scheiflinger, F., Dorner, F., and Falkner, F. G. [1998] "The complete genomic sequence of the modified vaccinia Ankara strain: Comparison with other orthopoxviruses" *Virology* 244:365-396) and 3kb in MCV (Senkevich, T. G., Koonin, E. V., Bugert, J. J., Darai, G., and Moss, B. [1997] "The genome of molluscum contagiosum virus: Analysis and comparison with other poxviruses" *Virology* 233:19-42). As seen in Table 1, each of the ORFs within the AmEPV ITR encodes a protein with no BLAST-derived function. The exception to this is AMVITR1. This most terminal gene has homology to MsEPV MSV010. Although this gene is not within the ITR region of the MsEPV genome, it is located towards the left terminus. The gene encodes a member of the leucine rich gene family protein.

Spontaneous DNA arrangements occur with an increased frequency at or near the terminal inverted repeat sequences of poxviral genomes (Moyer, R. W., Graves, R. L., and Rothe, C. T. [1980] "The white pock (mu) mutants of rabbit poxvirus. III. Terminal DNA sequence duplication and transposition in rabbit poxvirus" *Cell* 22:545-553). Indeed, the

majority of novel and non-essential genes are generally found within poxviral ITRs or toward the genomic termini.

AmEPV and vertebrate poxvirus gene homologs

The complete genomic sequences of vaccinia and variola viruses from the orthopoxvirus genus, myxoma and Shope fibroma viruses from the leporipoxvirus genus, fowlpox from the avipoxvirus genus, the molluscipoxvirus molluscum contagiosum and the genus B EPV, MsEPV have allowed definition of conserved poxvirus genes present in most, if not all, poxviruses. Inclusion of the AmEPV genomic sequence extends that concept.

Inspection of the AmEPV sequence shows 52 ORFs which have homology to genes found in ChPV (Table 2). Of these, 44 have been assigned a function. Of the 44 ORFs with an assigned function, 18 are derived from proteins involved in mRNA synthesis which include 5 ORFs comprising an RNA polymerase, 4 ORFs likely to encode transcription factors, 3 ORFs related to helicases/NTPases and 5 ORFs devoted to post-transcriptional mRNA modifications. Of the ORFs devoted to mRNA modification, the poly(A) polymerase deserves special mention. Normally, this heterodimeric enzyme consists of a large and small subunit. However, the AmEPV sequence reveals the presence of three rather than the expected two potential subunits. The ORFs AMV038, AMV060 and AMV115 are predicted to represent one large and two small poly(A) polymerase subunits respectively. This unusual feature will be discussed in a subsequent section. There are 7 homologous ORFs involved in functions of DNA replication/repair, which include a DNA polymerase, photolyase, nucleotide phosphohydrolase, DNA topoisomerase and a uracil DNA glycosylase. Interestingly, neither AmEPV nor MsEPV encode a homolog of the vaccinia I3L protein. The I3L protein is a DNA binding protein and is presumably involved in DNA replication (Davis, R. E. and Mathews, C. K. [1993] "Acidic C terminus of vaccinia virus DNA-binding protein interacts with ribonucleotide reductase" *Proc. Natl. Acad. Sci. U.S.A.* 90:745-749). Ten ChPV/EPV ORFs are associated with conserved virus structural proteins. Finally, there are 8 ORFS associated with enzymatic activities not strictly related to nucleic acid metabolism.

Table 2. Chordopoxvirus homologs found within AmEPV.

AmEPV ORF	Length amino acids	MsEPV ORF	Length amino acids	VV ORF	Length amino acids	Gene name and/or function
Transcription/RNA Modification						
RNA polymerase						
AMV051	349	149	348	A29L	305	RPO35
AMV054	822	119	807	H4L	795	RAP94
AMV066	1196	155	1190	A24R	1164	RPO132
AMV166	237	100	230	A5R	164	RPO19
AMV221	1301	43	1319	J6R	1286	RPO147
AMV230	179	245	186	D7R	161	RPO18
Transcription Factors						
AMV047	259	187	261	A1L	150	VLTF-2
AMV091	356	52	345	A23R	382	transcription factor
AMV105	767	63	760	A7L	710	VETF-L
AMV174	670	113	674	D6R	637	VETF-s
AMV205	228	65	218	A2L	224	VLTF-3
NTPase/helicase						
AMV059	469	148	471	A18R	493	DNA helicase
AMV081	720	86	717	I8R	676	RNA helicase
AMV192	648	53	647	D11L	631	NPH-I
mRNA modification						
AMV038	573	143	571	E1L	479	PAP-L
AMV060	295	41	293	J3R	333	PAP-S
AMV093	262	124	267	D12L	287	mRNA capping small subunit
AMV115	293	41	295	J3R	333	PAP-S
AMV135	627	67	860	D1R	844	mRNA capping large subunit
DNA replication/repair						
AMV016	182	N/A	N/A	J2R	177	Thymidine Kinase
AMV025	453	235	466	S127L*	445	CPD photolyase
AMV050	1105	36	964	E9L	1006	DNA polymerase
AMV052	333	130	328	H6R	314	DNA topoisomerase
AMV058	276	150	289	D10R	248	NTP pyrophosphohydorlase/ mutT
AMV087	726	89	834	D5R	785	NTPase
AMV231	344	208	232	D4R	218	uracil DNA glycosylase UNG
Structural						
AMV035	336	121	333	G9R	340	membrane protein
AMV061	255	158	293	L4R	251	30K virion protein
AMV118	386	90	380	A16L	378	membrane protein
AMV122	567	69	584	D13L	551	rifampicin resistance gene
AMV139	1149	152	1306	A10L	891	P4a core protein

AmEPV ORF	Length amino acids	MsEPV ORF	Length amino acids	VV ORF	Length amino acids	Gene name and/or function
AMV147	668	164	648	A3L	644	P4b core protein
AMV181	464	189	464	I7L	423	core protein
AMV217	246	183	242	L1R	250	myristylated membrane protein
AMV232	140	142	139	J5L	133	membrane protein
AMV243	248	94	241	F9L	212	membrane protein
Enzymes						
AMV078	165	N/A	N/A	S069L*	173	protein tyrosine phosphatase
AMV114	105	93	107	E10R	95	put. redox
AMV133	287	48	288	M5L**	75	lipase
AMV150	240	171	244	A32L	300	ATP/GTP binding protein
AMV153	468	173	457	F10L	439	Ser/Thr protein kinase
AMV197	299	154	396	B1R	300	Ser/Thr protein kinase
AMV256	609	56	629	G1L	591	metalloprotease
AMV255	152	N/A	N/A	A45R	163	Cu-Zn superoxide dismutase
Others						
AMV041	213	39	193	G6R	165	
AMV069	348	180	343	L3L	350	
AMV127	195	60	194	H2R	189	
AMV138	320	151	313	A11R	318	
AMV162	163	106	163	A22R	176	
AMV179	416	115	505	G5R	434	
AMV186	161	132	161	A28L	146	
AMV249	112	209	113	A21L	117	

All ChPV homolog ORF's shown are from VV. Where no homolog exists, *SFV, and **CPV.

Several of the genes included in Table 2 showing AmEPV vertebrate poxvirus homologs are not universally conserved, but are nevertheless present in many poxviruses. One example is the thymidine kinase (TK) gene. AmEPV encodes a TK gene, as do most ChPV and most other genus B EPVs investigated to date (Lytvyn, V., Fortin, Y., Banville, M., Arif, B., and Richardson, C. [1992] "Comparison of the thymidine kinase genes from three entomopoxviruses" *J. Gen. Virol.* 73:3235-3240). However, the gene is noticeably absent from both molluscum contagiosum and MsEPV (Senkevich, T. G., Koonin, E. V., Bugert, J. J., Darai, G., and Moss, B. [1997] "The genome of molluscum contagiosum virus: Analysis and comparison with other poxviruses" *Virology* 233:19-42; Afonso, C. L., Tulman, E. R., Lu, Z., Oma, E., Kutish, G. F., and Rock, D. L. [1999] "The genome of

Melanoplus sanguinipes Entomopoxvirus" *J. Virol.* 73, 533-552). Perhaps as previously suggested in the case of MsEPV, the absence of a TK and other enzymes related to nucleotide biosynthesis is reflective of a differential dependence on host biosynthetic pathways (Afonso *et al.* [1999] *supra*). Similarly, the CPD photolyase is not universally conserved within all members of the poxvirus family, but is present in a number of different viruses. Also of note is MsEPV ORF237, which is homologous to vaccinia virus B2R (Afonso *et al.* [1999] supra; Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. [1990] "The complete DNA sequence of vaccinia virus" *Virology* 179, 247-66, 517-63). This ORF, found at the right termini of both viruses, is absent from the genome of AmEPV. Likewise, a Cu/Zn superoxide dismutase (SOD) found within AmEPV (AMV255) is absent from the genome of MsEPV, and is fragmented or partially deleted in many orthopoxviruses (Smith, G. L., Chan, Y. S., and Howard, S. T. [1991] "Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the right inverted terminal repeat" *J. Gen. Virol.* 72:1349-1376;Cameron,C., Hota-Mitchell,S., Chen, L., Barrett, J., Cao, J. X., Macaulay, C., Willer, D., Evans, D., and McFadden, G. [1999] "The complete DNA sequence of myxoma virus" *Virology* 264:298-318). Both EPVs also encode a homolog of the A21L protein of VV. The A21L protein has been shown to interact with the A6L protein using the two hybrid system (McCraith, S., Holtzman, T., Moss, B., and Fields, S. [2000] "Genome-wide analysis of vaccinia virus protein-protein interactions" *Proc. Natl. Acad. Sci. U.S.A* 97:4879-4884). Interestingly, there is no homolog of the A6L protein in either EPV. Therefore, with the exception of the TK, SOD, protein tyrosine phosphatase and VV B2R, AmEPV and MsEPV share the same suite of ChPV virus homologs.

<u>A comparison of ORF content between AmEPV and other EPVs</u>

As well as the core poxviral genes shared between ChPV and EPV shown in Table 2, there are a number of genes which are shared between sequenced entomopoxviruses ie. AmEPV and MsEPV. Limited sequence data is also available from various regions of other entomopoxviruses currently under investigation. Given the vastly differing host

requirements of the ChPVs and EPVs, it is not unexpected that many genes differ between the two subfamilies. Approximately one third of genes encoded by ChPVs are responsible for a response against host immune defense systems (Gooding, L. R. [1992] "Virus proteins that counteract host immune defenses" *Cell* 71:5-7; Smith, G. L. [1994] "Virus strategies for evasion of the host response to infection" *Trends in Microbiol.* 2:81-88; Smith, G. L. [2000] "Secreted poxvirus proteins that interact with the immune system" *Effects of Microbes on the Immune System* 491-507). The 69 genes shared between MsEPV and AmEPV (but absent in ChPV's) are likely involved in insect specific interactions. The pattern of gene organization within the genome of Genus B EPVs has long been realized to be distinct from those of the ChPVs (Hall, R. L. and Moyer, R. W. [1991] "Identification, cloning, and sequencing of a fragment of *Amsacta moorei* entomopoxvirus DNA containing the spheroidin gene and three vaccinia virus-related open reading frames" *J. Virol.* 65:6516-6527; Sriskantha *et al.* [1997] *supra*; Afonso *et al.* [1999] *supra*). However it is now also evident that within genus B, obvious reorganization has occurred. For example, the NPH-1 and spheroidin homologs are immediately adjacent in all other known genus B viruses from *Choristoneura* and *Heliothis*, but are separated by 20kb in MsEPV. Similarly, the juxtaposed A23R protein and NPH-1 homolog in MsEPV are separated by 78kb in the AmEPV genome. Although there are no areas of organizational identity between the MsEPV and AmEPV genomes, there is one region of AmEPV genes (AMV159-AMV164) which contains homologs to MsEPV genes in the order of MSV111, 110, 108, 106, 112, 107. Given the lack of spatial conservation and degree of gene shuffling between MsEPV and AmEPV genes in all other areas of the AmEPV genome, small groups of genes may be present as the last remnants of divergence from a common ancestor. Alternatively, small clusters might have remained in close proximity to each other due to a more recent acquisition or for functional or regulatory reasons. Albeit not as striking an example, the homologs of MsEPV genes MSV085-MSV089 are also non-sequentially grouped within a 9 gene assembly within AmEPV (AMV079-AMV087). For these reasons, it is likely a conserved colinear core of genes may only be shared among the lepidopteran viruses within EPV genus B (Afonso *et al.* [1999] *supra*).

In this regard, the comparative alignment of two lepidopteran group B viruses, AmEPV and published HaEPV genes, does reveal some organizational similarities. Certain co-linear regions do appear to be shared. Positions of the spheroidin, NPH-1, "Q3" and DNA polymerases are all similarly situated within the genomes of the two viruses. The juxtaposed HaEPV PAP2, 30K and ORF4 genes are also immediately adjacent and co-linear in AmEPV, with ORF direction preserved (AMV060, AMV061 and AMV062)(Crnov and Dall 1999). Comparative alignments have also highlighted differences between these two more closely related genus B lepidopteran EPVs. For example, the large RNA polymerase of HaEPV is located toward the leftmost end of the genome, whereas it is positioned at the right end of AmEPV. Likewise the "17K" ORF of HaEPV is duplicated and teminally located within its ITRs, but homologous regions within AmEPV are not repeated, and are positioned approximately one hundred genes from the genomic termini. Whether or not a generally co-linear arrangement of genes emerges for the lepidopteran EPVs, it is obvious that EPVs in general have not followed the evolutionary direction of ChPV which has enabled them to retain a common co-linear gene core.

Clearly, genes shared between AmEPV and MsEPV are not arranged in a co-linear fashion and based on overall gene organization, MsEPV and AmEPV may be far more distantly related than the current common morphologically based classification as genus B EPVs would suggest. There are two possibilities to explain this divergence in gene order between AmEPV (lepidopteron) and MsEPV (orthopteran) viruses. One model employs a large evolutionary gap between the two viruses. A second model is based on intrinsic genomic plasticity and generalized movement of genes within the viral chromosome of EPVs. Comparative homologies among essential genes; *e.g.* RNA polymerase subunits, suggests MsEPV and AmEPV are more closely related to each other than either is to ChPVs homologs. Therefore, it may well be that plasticity or position independent location of genes within EPVs plays a significant role in the creation of divergent gene orders.

AmEPV encodes an additional 27 gene homologs not found within ChPV or MsEPV, but which are present in other insect viruses including baculoviruses (AcNPV, XcGV, SeNPV, CpGV, LdNPV and TnGV) and an iridovirus (Chilo iridescent virus). The majority

of these genes have previously been assigned functions, and a number are not specific to insect viruses alone (see Table 1).

AmEPV gene families

MsEPV was found to encode 43 novel ORFs which could be grouped into five gene families of varying stringency. Examination of the AmEPV genomic sequence revealed the presence of 23 genes which can be grouped into six gene families (Table 3).

Table 3. AmEPV Gene Families.

Gene family	AMV ORF	Size (aa)	Homology
AMV716	AMV056	86	none
	AMV176	86	none
	AMV178	86	none
ALI-like	AMV055	133	HaEPV ORF6/MSV194
	AMV057	352	HaEPV ORF6/MSV194
	AMV175	346	HaEPV ORF6/MSV194
	AMV177	360	HaEPV ORF6/MSV194
	AMV257	125	MSV196
MTG-like	AMV194	472	MSV198
	AMV207	476	MSV198
	AMV209	463	MSV198
Tryptophan	AMV029	290	MSV027
	AMV254	215	MSV027
17K ORF	AMV024	354	HaEPV 17K ORF/FPV124
	AMV110	362	HaEPV 17K ORF/FPV124
	AMV112	348	HaEPV 17K ORF/FPV124
	AMV100	136	HaEPV 17K ORF/FPV248
	AMV132	207	HaEPV 17K ORF/FPV248
LRR	AMVITR1	460	MSV010
	AMV005	350	MSV011
	AMV014	486	MSV240
	AMV076	117	MSV255
	AMV134	535	MSV240

The AMV176 gene family has no homology to any proteins within current databases. Each of these 86 residue proteins is identical, except for a single nucleotide substitution in AMV056 which results in an isoleucine codon at residue 37, instead of the leucine coded by

both AMV176 and AMV178. It is unusual to observe perfect copies of genes within a gene family. All members of the family are predicted to contain a transmembrane domain.

The five member ALI-like (alanine-leucine-isoleucine) gene family largely comprises ORFs related to the AMV 176 gene family discussed above. The ORFs do not possess any motifs indicative of transmembrane domains or signal sequences. AMV055, appears to be a carboxy terminal truncated member of this family. This 133 residue ORF shares a large number of residue identities with the other family members. The final member of the family, AMV257, appears to be truncated at the N-terminus, and is less related to the other members of this family. Nevertheless, its homology to MSV196 warrants its inclusion in this group.

A third MTG-like gene family has three members; AMV194, AMV207 and AMV209. There is a 69% identity between AMV207 and AMV209. AMV194 is somewhat less related to the other family members. Each gene was identified independantly based on its homology to the MTG gene family ORF MSV198 found in MsEPV. However, the invariant signature MTG (methionine-threonine-glycine) motif is absent from all AmEPV proteins, and an expected internal motif found within the MsEPV proteins was found to be degenerate.

A fourth family comprising only AMV029 and AMV254 shows homology to MsEPV ORF MSV027, which is a member of the tryptophan repeat gene family. Both AmEPV ORFs contain the expected motifs, although AMV029 does show degeneracy.

The fifth, 17K ORF gene family, contains five members which do not show any homology to MsEPV proteins, but are instead related to the 17K ORF of HaEPV. AMV024, AMV110 and AMV112 show excellent conservation at both their amino and carboxy termini, with a 60 residue internal portion of lesser similarity. Interestingly, these three genes also show homology with the N1R/p28 gene family of FPV (FPV124). AMV100 and AMV132 are also homologous to the HaEPV 17K ORF, and to FPV248, but less so. There is no homology between these two predicted AmEPV proteins themselves. Fifteen residues are shared between all members of this family.

The sixth gene family is the LRR (leucine-rich repeat) gene family which contains five AmEPV genes based upon the position of a motif containing regularly spaced leucine residues. There is a large LRR gene family in MsEPV. Each of the five members of the AmEPV LRR-like family shows homology to an LRR gene family protein of MsEPV. AMVITR1 and AMV005 are 63% identical, and very well conserved at their amino terminus. AMV014 and AMV134 share regions of homology along their lengths. At 117 residues, AMV076 is significantly smaller than other LRR-like gene family members (varying from 350 to 535 residues). However, when aligned with all other family members, an internal conserved motif emerges which includes seven leucine or isoleucine residues.

AmEPV ORFs encoding unique gene products

The majority of the unique AmEPV genes are located at the terminal extremes of the virus genome, as can be easily observed in Figure 7. More than one third of AmEPV ORFs (128 out of 279) show no homology to any sequences currently in the databases. We have classified these novel ORFs on the basis of whether they contain a predicted transmembrane domain (TM) and/or signal peptide (SP). Based on this classification, 4 ORFs possess predicted TM and SP domains, 3 an SP only, 56 a TM alone, and 65 possess neither. We expect that like the ChPV, a number of AmEPV genes are devoted to overcoming host defense responses.

Most genes encoded by AmEPV have homologs in ChPVs, EPVs or other insect viruses. In addition, there are a number of ORFs unique to AmEPV. There are also ORFs of interest potentially involved in host pathogenesis or virulence, such as AMV133 (SEQ ID NO: 1) which encodes a lipase, AMV255 (SEQ ID NO: 2) which encodes a superoxide dismutase (SOD), AMV025 (SEQ ID NO: 3) encoding a CPD photolyase and AMV021 (SEQ ID NO: 4) which encodes a baculovirus-like inhibitor of apoptosis (IAP). The following paragraphs briefly discuss each of these genes and their expected interactions with the host immune system.

AMV133 (SEQ ID NO: 1) encodes a AmEPV triacylglyceride lipase gene which could conceivably function as a virulence gene through lipid hydrolysis. AmEPV has been

shown to launch a promiscuous infection within the insect, including the fat body (Arif, B. M. and Kurstak, E. [1991]. The Entomopoxviruses. *In* "Viruses of Invertebrates," E. Kurstak, Ed.,, pp. 175-195. Marcel Dekker, Inc., New York), which is the major site of lipid storage (Chapman, R. F. [1998] Circulatory system, blood and immune systems. *In* "The Insects" pp. 94-131, Cambridge University Press, Cambridge). Although AmEPV infected insects do not undergo the "melting" phenotype associated with baculovirus infection, lipid hydrolysis would be anticipated to increase viral virulence, as has also been suggested for the lipase gene of MsEPV (Afonso *et al.* [1999] *supra*). Ectromelia and CPV are the only other poxviruses which encode similar proteins, and these are thought to play a role in the viral inflammatory response (Wall, E. M., Cao, J. X., Chen, N. H., Buller, R. L., and Upton, C. [1997] "A novel poxvirus gene and its human homolog are similar to an E-coli Lysophospholipase" *Virus Res.* 52:157-167).

AmEPV AMV255 (SEQ ID NO: 2) encodes a Cu^{++}/Zn^{++} superoxide dismutase homolog. These proteins are widespread in nature and are recognized as a primary defense against the damage of superoxide radicals (Fridovich, I. [1997] "Superoxide anion radical (O2-.), superoxide dismutases, and related matters" *J. Biol. Chem.* 272:18515-18517). Although the SOD homolog was initially discovered in a baculovirus (Tomalski, M. D., Eldridge, R., and Miller, L. K. [1991] "A baculovirus homolog of a Cu/Zn superoxide dismutase gene" *Virology* 184, 149-161), all sequenced ChPVs have also been found to encode a vestige of a SOD. However, many include deletions or substitutions within the coding region which render the protein inactive (Smith, G. L., Chan, Y. S., and Howard, S. T. [1991] "Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the right inverted terminal repeat" *J. Gen. Virol.* 72, 1349-1376; Willer *et al.* [1999] *supra*) but the AmEPV homolog appears to be intact. MsEPV does not encode a *sod* (Afonso *et al.* [1999] *supra*).

During their life cycle, most insect viruses spend some period of time exposed to potentially detrimental environmental conditions. Therefore it is somewhat surprising that more insect viral genomes do not contain light-dependant DNA-repair mechanisms. AmEPV AMV025 (SEQ ID NO: 3) and MsEPV both encode a CPD photolyase homolog,

as do the ChPV SFV and MYX. These are the only reports of virally encoded CPD photolyases.

Viruses have evolved various strategies to inhibit apoptosis, thereby allowing intracellular viral replication. In ChPVs, apoptosis is controlled in part by serpins (Petit, F., Bergagnoli, S., Gelfi, J., Fassy, F., Boucraut-Baralon, C., and Milon, A. [1996] "Characterization of a myxoma virus-encoded serpin-like protein with activity against interleukin-1b converting enzyme" *J. Virol.* 70:5860-5866; Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R., Salvesen, G. S., and Pickup, D. J. [1992] "Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme" *Cell* 69:597-604; Spriggs, M. K., Hruby, D. E., Maliszeswki, C. R., Pickup, D. J., Sims, J. E., Buller, R. M. L., and VanSlyke, J. [1992] "Vaccinia and Cowpox viruses encode a novel secreted interleukin-1 binding protein" *Cell* 71:145-152; Ray, C. A. and Pickup, D. J. [1996] "The mode of death of pig kidney cells infected with cowpox virus is governed by the expression of the crmA gene" *Virology* 217:384-391; Macen, J., Takahashi, A., Moon, K. B., Nathaniel, R., Turner, P. C., and Moyer, R. W. [1998] "Activation of caspases in pig kidney cells infected with wild-type and CrmA/SPI-2 mutants of cowpox and rabbitpox viruses" *J. Virol.* 72:3524-3533; Turner, P. C. and Moyer, R. W. [1998] "Control of apoptosis by poxviruses. *Seminars in Virology* 8:453-469). Insect viruses control apoptosis through either p35 or through a series of inhibitor of apoptosis (IAP) proteins (Deveraux, Q. L. and Reed, T. C. [1999] "IAP family proteins - suppressors of apoptosis" *Genes & Development* 13:239-252; Miller, L. K. [1999] "An exegesis of IAPs: salvation and surprises from BIR motifs" *Trends Cell Biol.* 9:323-328; Manji, G. A., Hozak, R. R., LaCount, D. J., and Friesen, P. D. [1997] "Baculovirus inhibitor of apoptosis functions at or upstream of the apoptotic suppressor P35 to prevent programmed cell death" *J. Virol.* 71:4509-4516). AMV021 (SEQ ID NO: 4) encodes one such inhibitor of apoptosis protein (IAP), and contains two typical baculovirus IAP repeats and a C-terminal RING finger motif. The AMV021 ORF shows significant identity to the IAP of *Cydia pomonella* granulosis virus (47%), which has previously been shown to be functionally active (Crook, N. E., Clem, R. J., and Miller, L. K. [1993] "An apoptosis-inhibiting baculovirus gene with a zinc finger-

like motif" *J. Virol.* 67:2168-2174). AmEPV and MsEPV are the only poxviruses found to encode IAPs. These proteins have only been noted in the genomes of viruses which infect insect or arthropod hosts.

The following are several different ORFs encoded by AmEPV, which are notable either because they currently do not have homologs in any published viral sequence to date or possess novel aspects of previously described poxvirus genes.

AMV060 (SEQ ID NO: 5) and AMV115 (SEQ ID NO: 6) encode a first and second AmEPV poly(A) polymerase subunit. ORFs AMV060 and AMV115 present a completely unanticipated variation of a well detailed poxvirus encoded enzyme, the poly(A) polymerase. The cytoplasmic synthesis of poxvirus mRNAs involves not only transcription of a given gene by the viral RNA polymerase, but also post-transcriptional modification of the transcripts, including 3' poly(A) addition and 5'capping as well as 2'O-methylation. In the case of VV, addition of poly(A) to transcripts and 2'O-methylation of the mRNAs involves a heterodimeric poly(A) polymerase consisting of one large (VP55) and one single small (VP39) subunit encoded by two distinct ORFs (Brakel, C. and Kates, J. R. [1974] "Poly(A) polymerase from vaccinia virus-infected cells. I. Partial purification and characterization" *J. Virol.* 14:715-723; Gershon, P. D., Ahn, B. Y., Garfield, M., and Moss, B. [1991] "Poly(A) polymerase and a dissociable polyadenylation stimulatory factor encoded by vaccinia virus" *Cell* 66:1269-1278; Schnierle, B. S., Gershon, P. D., and Moss, B. [1992] "Cap-specific mRNA (nucleoside-O2'-)-methyltransferase and poly(A) polymerase stimulatory activities of vaccinia virus are mediated by a single protein" *Proc. Natl. Acad. Sci. U.S.A.* 89:2897-2901).

The AmEPV genomic sequence has revealed an unusual feature of the poly(A) polymerase in this entomopoxvirus. Like other poxviruses, there is a single, large subunit (AMV038) (SEQ ID NO: 11) of approximately 570 amino acids. This is similar in size to the large VV poly(A) polymerase subunit (VP55). However, unlike any other poxvirus (Afonso *et al.* [2000] *supra*; Afonso *et al.* [1999] *supra*; Cameron *et al.* [1999] *supra*; Willer *et al.* [1999] *supra*; Senkevich *et al.* [1997] *supra*; Antoine *et al.* [1998] *supra*; Goebel *et al.* [1990] *supra*), sequencing suggests that AmEPV may encode two small subunits (AMV060

and AMV115). The two small subunits are somewhat smaller than the 333 amino acid VV small subumit (295 and 293 amino acids respectively) (Figure 10) and related throughout their length.

Comparison of both AMV060 and AMV115 to the small subunit of VV (VP39, ORF J3R), MsEPV (MSV041) and the sole poly(A) polymerase subunit revealed in the incompletely sequenced genome of HaEPV, is striking (Figure 10). Both AmEPV small subunits show the largest degrees of relatedness to other poxvirus poly(A) polymerase small subunits within the first 200 amino acids. Both AmEPV small subunits contain a highly conserved poly(A) polymerase regulatory structural motif encompassing amino acids 1-281 within AMV060 and amino acids 8-271 within AMV115. The AMV060 subunit is more related to VP39 than is AMV115. However, if both the two AmEPV small subunits are both compared to the single poly(A) polymerase small subunit of MsEPV (MSV041), the homologies for both AMV60 and AMV115 to MSV041 comparable and greater than either of the small AmEPV subunits to VV. BLAST values for AMV060 showed it to be most related to the small poly(A) polymerase subunit sequenced from HaEPV (Sriskantha *et al.* [1997] *supra*), while AMV115 was most homologous to that of MsEPV.

One highly conserved, ungapped motif (Figure 10) [L/V]-Y-I-G-S-X-X-[G/A]-[Y/T]-H-X-X-X-L can be somewhat expanded if comparisons are limited to only EPV sequences. Note that there are other completely conserved residues in the centermost region of the proteins, and many other conservative substitutions.

Another interesting feature is revealed when one examines the C-terminus of the small subunits (Figure 10). One immediately notes that the comparable VV small subunit contains a C-terminal extension. The VV 36-43 amino acid C-terminal tail is non-essential for activity (Shi, X., Yao, P., Jose, T., and Gershon, P. [1996] "Methyltransferase-specific domains within VP-39, a bifunctional protein that participates in the modification of both mRNA ends" *RNA* 2:88-101), and is probably retained because the C-terminal region of the VV subunit overlaps the next open reading frame (J4R) which encodes a 22kDa subunit of the VV RNA polymerase (Goebel *et al.* [1990] *supra*).

One functional hypothesis to account for the presence of an additional small poly(A) subunit is suggested by the multiple activities of the poly(A) polymerase itself (Gershon, P. D., Shi, X. N., and Hodel, A. E. [1998] "Evidence that the RNA methylation and poly(A) polymerase stimulatory activities of vaccinia virus protein VP39 do not impinge upon one another" *Virology* 246:253-265). VV VP55 catalyzes the initial (~35 base) addition of 3' poly(A) to newly synthesized mRNA or 5' phosphorylated nucleotide primers (Gershon, P. D., Ahn, B. Y., Garfield, M., and Moss, B. [1991] "Poly(A) polymerase and a dissociable polyadenylation stimulatory factor encoded by vaccinia virus" *Cell* 66:1269-1278). The small subunit, VP39 has three activities. The first is to serve as a processivity factor which in the presence of VP55 extends the poly(A) length to several hundred A residues (Gershon, P. D. and Moss, B. [1993] "Stimulation of poly(A) tail elongation by the VP39 subunit of the vaccinia virus-encoded poly(A) polymerase" *J. Biol. Chem.* 268:2203-2210). The second, distinct activity, mediated by VP39 alone, is an mRNA cap-specific 2'-O-methyltransferase (Schnierle *et al.* [1992] *supra*). The third activity is an associated transcription elongation factor (Latner, D. R., Xiang, Y., Lewis, J. I., Condit, J., and Condit, R. C. [2000] "The vaccinia virus bifunctional gene J3 (nucleoside-2 '-O-)-methyltransferase and poly(A) polymerase stimulatory factor is implicated as a positive transcription elongation factor by two genetic approaches" *Virology* 269:345-355). It is possible that these various activities have been distributed amongst the two subunits. Alternatively, one of the subunits may have evolved to fulfill an entirely unrelated function.

AMV050 (SEQ ID NO: 7) and AMV210 (SEQ ID NO: 8) encode AmEPV DNA polymerases. In view of our findings with the poly(A) polymerase, we would like to call attention to an interesting feature of EPV DNA polymerases first noted in African Swine Fever virus (Oliveros, M., Yanez, R. J., Salas, M. L., Salas, J., Vinuela, E., and Blanco, L. [1997] "Characterization of an African swine fever virus 20-kDa DNA polymerase involved in DNA repair" *J. Biol. Chem.* 272:30899-30910) and later in MsEPV (Afonso *et al.* [1999] *supra*), which has also been found in AmEPV. The 1105 residue AmEPV ORF AMV050 is similar in length, and homologous to typical poxvirus encoded DNA polymerases. A second smaller (612 amino acids) AmEPV encoded ORF, AMV210, shares a 460 amino acid region

of clear homology with AMV050, although both proteins possess completely unique regions; i.e., the N-terminus of AMV050 (residues 1-645) and C-terminus of AMV210 (residues 463-612). Both proteins have been found to contain DNA polymerase motifs (Table 1).

AMV130 (SEQ ID NO: 9) encodes an AmEPV ABC transporter-like protein. AMV130 represents the largest ORF in AmEPV. The 1384 residue protein shows homology to the ATP-binding cassette (ABC) proteins. These are a large gene family found from bacteria to man, and have a variety of functions (van Veen, H. W. and Konings, W. N. [1998] "Structure and function of multidrug transporters" *Adv. Exp. Med. Biol.* 456:145-158). While most are ATP-driven membrane translocators, some act as ion channels, ion channel regulators, receptors, proteases, immune regulators and even sensing proteins (Bauer, B. E., Wolfger, H., and Kuchler, K. [1999] "Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance" *Biochim. Biophys. Acta* 1461:217-236; Klein, I., Sarkadi, B., and Radi, A. [1999] "An inventory of the human ABC proteins" *Biochim. Biophys. Acta* 1461:237-262; Abele, R. and Tampe, R. [1999] "Function of the transport complex TAP in cellular immune recognition" *Biochim. Biophys. Acta* 1461:405-419). All ABC proteins share a common molecular architecture consisting of at least one 200-250 amino acid ABC cassette and several predicted α-helical membrane spanning segments (TMS or TMD). The minimum structural requirement is considered to be 2 ABC and 2 TMD regions, present in either 1 (full transporter) or 2 (half transporter) polypeptide chains. The AmEPV ABC protein consists of TMD-ABC-TMD-ABC domains, one of the structures of active ABC transporters. This arrangement of AMV130 domains is also found in the MDR/TAP, MRP, CFTR and ABC1 subfamilies and is associated with activities ranging from control of sex (yeast), drug resistance (humans, bacteria), ion channels (human CFTR gene) and engulfment of dead cells (*C. elegans*) (Bauer *et al.* [1999] *supra*; Klein *et al.* [1999] *supra*; Abele and Tampe [1999] *supra*). Each AmEPV TMD contains 6 or 7 transmembrane helices (Figure 11). No other virus is known to encode an ABC transporter. The potential ABC-transporter encoded by AmEPV may play a role in evading host immune defenses, e.g. facilitating removal of toxic elements from virally infected cells.

AMV007 (SEQ ID NO: 10) encodes an AmEPV Kunitz-motif protease inhibitor (KPI). AmEPV ORF AMV007 is located near the left end of the AmEPV genome, and encodes a small protein of 79 amino acids. A Prosite search revealed the presence of a Kunitz family signature (Prosite PS00280), a motif associated with protease inhibitors (Figure 12). Indeed, the Kunitz-type pancreatic trypsin inhibitors represent one of the most common families of serine protease inhibitors. Kunitz-type inhibitors found within insects are typically less than 100 amino acids in length. All contain certain five invariant cysteine residues. AMV007 has all five cysteines and the alignment allows prediction of an arginine P1. The inducible serine protease inhibitor (ISP-2) of *Galleria mellonella* (Frobius, A. C., Kanost, M. R., Gotz, P., and Vilcinskas, A. [2000] "Isolation and characterization of novel inducible serine protease inhibitors from larval hemolymph of the greater wax moth Galleria mellonella" *Eur. J. Biochem.* 267:2046-2053) and the hemolymph trypsin inhibitors (HLTIs A and B) of *Manduca sexta* (Ramesh, N., Sugumaran, M., and Mole, J. E. [1988] "Purification and characterizationof two trypsin inhibitors from the hemolymph of Manduca sexta larvae" *J. Biol. Chem.* 263:11523-11527) are both Kunitz-type inhibitors that contain P1 residues of arginine, and inhibit trypsin-like proteases. Structurally, Kunitz-type inhibitors are comprised of short alpha/beta proteins with little secondary structure. Although widespread in nature, there are no reports of the presence of a Kunitz-type protease inhibitor (KPI) from this family in any viral genome. It is interesting to note that vertebrate poxviruses do encode protease inhibitors, but they are members of a different family (the serine protease inhibitor, serpin) family. The vertebrate poxvirus serpins have been shown to have an immunoregulatory role in the infected vertebrate host (Turner, S., Kenshole, B., and Ruby, J. [1999] "Viral modulation of the host response via crmA/SPI-2 expression" *Immunology and Cell Biology* 77:236-241; McFadden, G. [1995] In: *Viroceptors, virokines and related immune modulators encoded by DNA viruses*, R. G. Landes/Springer-Verlag, Austin, TX; McFadden, G., Graham, K., and Barry, M. [1996] "New Strategies of immune modulation by DNA viruses" *Transplant. Proc.* 28:2085-2088). We propose that the AmEPV KPI protein may fulfill a similar immunoregulatory role in the infected invertebrate host, but may target different pathways than do the serpins which control inflammation,

apoptosis and the host immune response (Turner and Moyer [1998] *supra*; Turner, P. C., Musy, P. Y., and Moyer, R. W. [1995] Poxvirus Serpins, In "Viroceptors, Virokines and related immune modulators encoded by DNA viruses," G. McFadden, ed., pp. 67-88. R. G. Landes, Galveston, TX).

One function the KPI protein may possess is suggested by the physiology of the insect host. The haemolymph of insects contains relatively high concentrations of a variety of protease inhibitors from several different gene families (Kanost, M. R. [1999] "Serine proteinase inhibitors in arthropod immunity" *Developmental and Comparative Immunology* 23:291-301; Jiang, H. B. and Kanost, M. R. [1997] "Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from Manduca sexta" *J. Biol. Chem.* 272:1082-1087). Protease inhibitors from the Kunitz family have been identified as haemolymph proteins from lepidopteran insect species (Sugumaran, M., Saul, S. J., and Ramesh, N. [1985] "Endogenous protease inhibitors prevent undesired activation of prophenolase in insect hemolymph" *Biochem. Biophys. Res. Commun.* 132:1124-1129; Sasaki, T. [1984] "Amino acid sequence of a novel Kunitz-type chymotrysin inhibitor from hemolymph of silkworm larvae" *Bombyx moorei. FEBS Lett.* 168:230), which function as inhibitors of trypsin or chymotrypsin. These host KPI proteins have been shown to be important in the avoidance of inopportune chymotrypsin-mediated activation of prophenyloxidase (Saul, S. J. and Sugumaran, M. [1986] "Protease inhibitor controls prophenoloxidase activation in Manduca sexta" *FEBS Lett.* 208:113-116; Aso, Y., Yamashita, T., Meno, K., and Murakami, M. [1994] "Inhibition of prophenoloxidase-activating enzyme from Bombyx mori by endogenous chymotrypsin inhibitors" *Biochem. Mol. Biol. Int.* 33:751-758). This enzyme is an early component of the cascade required by the insect immune system to produce melanin, which is used to engulf and overcome invading foreign objects (Gillespie, J. P., Kanost, M. R., and Trenczek, T. [1997] "Biological mediators of insect immunity" *Annu. Rev. Entomol.* 42:611-43, 611-643; Vilmos, P. and Kurucz, E. [1998] "Insect immunity: evolutionary roots of the mammalian innate immune system" *Immunol. Lett.* 62:59-66). Production of such a protein by an infecting virus may

therefore lessen the amount of prophenyloxidase induced by the insect immune system during infection.

Polynucleotides of the subject invention include sequences identified in the attached sequence listing as well as the tables and figures and described by open reading frame (ORF) position within the genome. In addition, the subject invention includes polynucleotides which hybridize with other polynucleotides of the subject invention.

Additional uses of polynucleotides. The polynucleotide sequences exemplified herein can be used in a variety of ways, having numerous applications in techniques known to those skilled in the art of molecular biology having the instant disclosure. These techniques include their use as hybridization probes, for chromosome and gene mapping, in PCR technologies, and in the production of sense or antisense nucleic acids.

These polynucleotides can be used in assays for additional polynucleotides and additional homologous genes, and can be used in tracking the quantitative and temporal expression of these genes in cells and organisms. Polynucleotides of the subject invention may be used as insertion sites for foreign genes of interest.

Antisense technology can also be used to interfere with expression of the disclosed polynucleotides. For example, the transformation of a cell or organism with the reverse complement of a gene encoded by a polynucleotide exemplified herein can result in strand co-suppression and silencing or inhibition of a target gene, *e.g.*, one involved in the infection process.

Polynucleotides disclosed herein are useful as target genes for the synthesis of antisense RNA or dsRNA useful for RNA-mediated gene interference. The ability to specifically inhibit gene function in a variety of organisms utilizing antisense RNA or ds RNA-mediated interference is well known in the fields of molecular biology (see for example C.P. Hunter, Current Biology [1999] 9:R440-442; Hamilton et al., [1999] Science, 286:950-952; and S.W. Ding, Current Opinions in Biotechnology [2000] 11:152-156, hereby incorporated by reference in their entireties). dsRNA (RNAi) typically comprises a polynucleotide sequence identical or homologous to a target gene (or fragment thereof) linked directly, or indirectly, to a polynucleotide sequence complementary to the sequence

of the target gene (or fragment thereof). The dsRNA may comprise a polynucleotide linker sequence of sufficient length to allow for the two polynucleotide sequences to fold over and hybridize to each other; however, a linker sequence is not necessary. The linker sequence is designed to separate the antisense and sense strands of RNAi significantly enough to limit the effects of steric hindrances and allow for the formation of dsRNA molecules and should not hybridize with sequences within the hybridizing portions of the dsRNA molecule. The specificity of this gene silencing mechanism appears to be extremely high, blocking expression only of targeted genes, while leaving other genes unaffected. Accordingly, one method for controlling gene expression according to the subject invention provides materials and methods using double-stranded interfering RNA (dsRNAi), or RNA-mediated interference (RNAi). The terms dsRNAi and RNAi are used interchangeably herein unless otherwise noted.

RNA containing a nucleotide sequence identical to a fragment of the target gene is preferred for inhibition; however, RNA sequences with insertions, deletions, and point mutations relative to the target sequence can also be used for inhibition. Sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, *Sequence Analysis Primer*, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (*e.g.*, University of Wisconsin Genetic Computing Group). Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a fragment of the target gene transcript.

RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or *in vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region (*e.g.*, promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands); the promoters may be known inducible promoters such as baculovirus. Inhibition may be targeted by specific transcription in an organ, tissue, or cell type. The RNA strands may or may not be

polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (*e.g.*, T3, T7, SP6). The use and production of an expression construct are known in the art (see, for example, WO 97/32016; U.S. Pat. Nos. 5,593,874; 5,698,425; 5,712,135; 5,789,214; and 5,804,693; and the references cited therein). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

Preferably and most conveniently, dsRNAi can be targeted to an entire polynucleotide sequence set forth herein. Preferred RNAi molecules of the instant invention are highly homologous or identical to the polynucleotides of the sequence listing. The homology may be greater than 70%, preferably greater than 80%, more preferably greater than 90% and is most preferably greater than 95%.

Fragments of genes can also be utilized for targeted suppression of gene expression. These fragments are typically in the approximate size range of about 20 nucleotides. Thus, targeted fragments are preferably at least about 15 nucleotides. In certain embodiments, the gene fragment targeted by the RNAi molecule is about 20-25 nucleotides in length. In a more preferred embodiment, the gene fragments are at least about 25 nucleotides in length. In an even more preferred embodiment, the gene fragments are at least 50 nucleotides in length.

Thus, RNAi molecules of the subject invention are not limited to those that are targeted to the full-length polynucleotide or gene. Gene product can be inhibited with a RNAi molecule that is targeted to a portion or fragment of the exemplified polynucleotides;

high homology (90-95%) or greater identity is also preferred, but not necessarily essential, for such applications.

In another aspect of the invention, the dsRNA molecules of the invention may be introduced into cells with single stranded (ss) RNA molecules which are sense or anti-sense RNA derived from the nucleotide sequences disclosed herein. Methods of introducing ssRNA and dsRNA molecules into cells are well-known to the skilled artisan and includes transcription of plasmids, vectors, or genetic constructs encoding the ssRNA or dsRNA molecules according to this aspect of the invention; electroporation, biolistics, or other well-known methods of introducing nucleic acids into cells may also be used to introduce the ssRNA and dsRNA molecules of this invention into cells.

Other aspects of the invention include use of the disclosed sequences or recombinant nucleic acids derived therefrom to produce purified peptides. The nucleotide sequences as disclosed herein may be used to produce an amino acid sequence using well known methods of recombinant DNA technology. Goeddel (Gene Expression Technology, Methods and Enzymology [1990] Vol 185, Academic Press, San Diego, CA) is one among many publications which teach expression of an isolated, purified nucleotide sequence. The amino acid or peptide may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from the same species from which the nucleotide sequence was derived or from a different species.

Still further aspects of the invention use these purified peptides to produce antibodies or other molecules able to bind to the peptides. These antibodies or binding agents can then be used for the screening of cells in order to localize the cellular distribution of the peptides or proteins. The antibodies are also useful for the affinity purification of recombinantly produced peptides or proteins.

The disclosed nucleotide sequences can be used individually, or in panels, in tests or assays to detect levels of peptide, polypeptide, or protein expression. The form of such qualitative or quantitative methods may include northern analysis, dot blot or other membrane based technologies, dip stick, pin or chip technologies, PCR, ELISAs or other multiple sample format technologies.

The subject invention also provides polynucleotides identified as control elements or regulatory sequences, such as gene promoters, enhancers, introns and untranslated regions which interact with cellular components to carry out regulatory functions such as replication, transcription, and translation. The invention further comprises the use of the disclosed polynucleotide sequences, or fragments thereof, in assays to characterize and/or identify sequences having promoter or other regulatory activity. Also contemplated according to the subject invention is the use of oligomers from these sequences in kits which can be used to identify promoters or other regulatory sequences.

As used herein, the following definitions apply:

An "oligonucleotide" or "oligomer" is a stretch of nucleotide residues which has a sufficient number of bases to be used in a polymerase chain reaction (PCR). These short sequences are based on (or designed from) genomic or cDNA sequences and are used to amplify, confirm, or reveal the presence of an identical, similar or complementary DNA or RNA in a particular cell or tissue. Oligonucleotides or oligomers comprise portions of a DNA sequence having at least about 10 nucleotides and as many as about 50 nucleotides, preferably about 15 to 30 nucleotides. They can be chemically synthesized and may be used as probes.

"Probes" are nucleic acid sequences of variable length, preferably between at least about 10 and as many as about 6,000 nucleotides, depending on use. They are used in the detection of identical, similar, or complementary nucleic acid sequences. Longer length probes are usually obtained from a natural or recombinant source, are highly specific and much slower to hybridize than oligomers. They may be single- or double-stranded and designed to have specificity in PCR, hybridization membrane-based, or ELISA-like technologies.

"Reporter" molecules are chemical moieties used for labeling a nucleic or amino acid sequence. They include, but are not limited to, radionuclides, enzymes, fluorescent, chemi-luminescent, or chromogenic agents. Reporter molecules associate with, establish the presence of, and may allow quantification of a particular nucleic or amino acid sequence.

A "portion" or "fragment" of a polynucleotide or nucleic acid comprises all or any part of the nucleotide sequence having fewer nucleotides than about 6 kb, preferably fewer than about 1 kb which can be used as a probe. Such probes may be labeled with reporter molecules using nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. After pretesting to optimize reaction conditions and to eliminate false positives, nucleic acid probes may be used in Southern, northern or in situ hybridizations to determine whether target DNA or RNA is present in a biological sample, cell type, tissue, organ or organism.

"Recombinant nucleotide variants" are alternate polynucleotides which encode a particular protein. They may be synthesized, for example, by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes which produce specific restriction sites or codon usage-specific mutations, may be introduced to optimize cloning into a plasmid or viral vector or expression in a particular prokaryotic or eukaryotic host system, respectively.

"Linkers" are synthesized palindromic nucleotide sequences which create internal restriction endonuclease sites for ease of cloning the genetic material of choice into various vectors. "Polylinkers" are engineered to include multiple restriction enzyme sites and provide for the use of both those enzymes which leave 5' and 3' overhangs such as BamHI, EcoRI, PstI, KpnI and Hind III or which provide a blunt end such as EcoRV, SnaBI and StuI.

"Control elements" or "regulatory sequences" are regions of the gene or DNA such as enhancers, promoters, introns and 3' untranslated regions which interact with cellular proteins to carry out replication, transcription, and translation. Typically, these regions are nontranslated. They may occur as boundary sequences or even split the gene. They function at the molecular level and along with regulatory genes are very important in development, growth, differentiation and aging processes.

"Chimeric" molecules are polynucleotides or polypeptides which are created by combining one or more nucleotide peptide sequences (or their parts). In the case of nucleotide sequences, such combined sequences may be introduced into an appropriate vector and expressed to give rise to a chimeric polypeptide which may be expected to be

different from the native molecule in one or more of the following characteristics: cellular location, distribution, ligand-binding affinities, interchain affinities, degradation/turnover rate, signaling, etc.

"Active" is that state which is capable of being useful or of carrying out some role. It specifically refers to those forms, fragments, or domains of an amino acid sequence which display the biologic and/or immunogenic activity characteristic of the naturally occurring peptide, polypeptide, or protein.

"Naturally occurring" refers to a polypeptide produced by cells which have not been genetically engineered or which have been genetically engineered to produce the same sequence as that naturally produced.

"Derivative" refers to those polypeptides which have been chemically modified by such techniques as ubiquitination, labeling, pegylation (derivatization with polyethylene glycol), and chemical insertion or substitution of amino acids such as ornithine which do not normally occur in proteins.

"Recombinant polypeptide variant" refers to any polypeptide which differs from naturally occurring peptide, polypeptide, or protein by amino acid insertions, deletions and/or substitutions.

Amino acid "substitutions" are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

Amino acid "insertions" or "deletions" are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. The variation allowed in a particular amino acid sequence may be experimentally determined by producing the peptide synthetically or by systematically making insertions, deletions, or substitutions of nucleotides in the sequence using recombinant DNA techniques.

A "signal or leader sequence" is a short amino acid sequence which can be used, when desired, to direct the polypeptide through a membrane of a cell. Such a sequence may be naturally present on the polypeptides of the present invention or provided from

heterologous sources by recombinant DNA techniques. Such sequences include nuclear localization sequences (NLS) known in the art.

An "oligopeptide" is a short stretch of amino acid residues and may be expressed from an oligonucleotide. Such sequences comprise a stretch of amino acid residues of at least about 5 amino acids and often about 17 or more amino acids, typically at least about 9 to 13 amino acids, and of sufficient length to display biologic and/or immunogenic activity.

An "inhibitor" is a substance which retards or prevents a chemical or physiological reaction or response. Common inhibitors include but are not limited to antisense molecules, antibodies, antagonists and their derivatives.

A "standard" is a quantitative or qualitative measurement for comparison. Preferably, it is based on a statistically appropriate number of samples and is created to use as a basis of comparison when performing diagnostic assays, running clinical trials, or following patient treatment profiles. The samples of a particular standard may be normal or similarly abnormal.

Since the list of technical and scientific terms cannot be all encompassing, any undefined terms shall be construed to have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. Furthermore, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

The invention is not to be limited only to the particular sequences, variants, formulations or methods described. The sequences, variants, formulations and methodologies may vary, and the terminology used herein is for the purpose of describing particular embodiments. The terminology and definitions are not intended to be limiting.

<u>Polynucleotide probes</u>. DNA possesses a fundamental property called base complementarity. In nature, DNA ordinarily exists in the form of pairs of anti-parallel strands, the bases on each strand projecting from that strand toward the opposite strand. The base adenine (A) on one strand will always be opposed to the base thymine (T) on the other strand, and the base guanine (G) will be opposed to the base cytosine (C). The bases are held in apposition by their ability to hydrogen bond in this specific way. Though each individual bond is relatively weak, the net effect of many adjacent hydrogen bonded bases, together

with base stacking effects, is a stable joining of the two complementary strands. These bonds can be broken by treatments such as high pH or high temperature, and these conditions result in the dissociation, or "denaturation," of the two strands. If the DNA is then placed in conditions which make hydrogen bonding of the bases thermodynamically favorable, the DNA strands will anneal, or "hybridize," and reform the original double- stranded DNA. If carried out under appropriate conditions, this hybridization can be highly specific. That is, only strands with a high degree of base complementarity will be able to form stable double-stranded structures. The relationship of the specificity of hybridization to reaction conditions is well known. Thus, hybridization may be used to test whether two pieces of DNA are complementary in their base sequences. It is this hybridization mechanism which facilitates the use of probes of the subject invention to readily detect and characterize DNA sequences of interest.

The polynucleotides of the subject invention can themselves be used as probes. Additional polynucleotide sequences can be added to the ends of (or internally in) the exemplified polynucleotide sequences so that polynucleotides that are longer than the exemplified polynucleotides can also be used as probes. Thus, isolated polynucleotides comprising one or more of the exemplified sequences are within the scope of the subject invention. Polynucleotides that have less nucleotides than the exemplified polynucleotides can also be used and are contemplated within the scope of the present invention. For example, for some purposes, it might be useful to use a conserved sequence from an exemplified polynucleotide wherein the conserved sequence comprises a portion of an exemplified sequence. Thus, polynucleotides of the subject invention can be used to find additional, homologous (wholly or partially) genes. Hybridization probes of the subject invention may be derived from the open reading frames specifically exemplified in the sequence listing, figures, and tables as well as from surrounding or included genomic sequences comprising untranslated regions such as promoters, enhancers and introns.

Probes of the subject invention may be composed of DNA, RNA, or PNA (peptide nucleic acid). The probe will normally have at least about 10 bases, more usually at least about 17 bases, and may have up to about 100 bases or more. Longer probes can readily be

utilized, and such probes can be, for example, several kilobases in length. The probe sequence is designed to be at least substantially complementary to a portion of a gene encoding a protein of interest. The probe need not have perfect complementarity to the sequence to which it hybridizes. The probes may be labeled utilizing techniques that are well known to those skilled in this art.

One approach for the use of the subject invention as probes entails first identifying DNA segments that are homologous with the disclosed nucleotide sequences using, for example, Southern blot analysis of a gene bank. Thus, it is possible, without the aid of biological analysis, to know in advance the probable activity of many new polynucleotides, and of the individual gene products expressed by a given polynucleotide. Such an analysis provides a rapid method for identifying commercially valuable compositions.

One hybridization procedure useful according to the subject invention typically includes the initial steps of isolating the DNA sample of interest and purifying it chemically. Either lysed cells or total fractionated nucleic acid isolated from cells can be used. Cells can be treated using known techniques to liberate their DNA (and/or RNA). The DNA sample can be cut into pieces with an appropriate restriction enzyme. The pieces can be separated by size through electrophoresis in a gel, usually agarose or acrylamide. The pieces of interest can be transferred to an immobilizing membrane.

The particular hybridization technique is not essential to the subject invention. As improvements are made in hybridization techniques, they can be readily applied.

The probe and sample can then be combined in a hybridization buffer solution and held at an appropriate temperature until annealing occurs. Thereafter, the membrane is washed free of extraneous materials, leaving the sample and bound probe molecules typically detected and quantified by autoradiography and/or liquid scintillation counting. As is well known in the art, if the probe molecule and nucleic acid sample hybridize by forming a strong non-covalent bond between the two molecules, it can be reasonably assumed that the probe and sample are essentially identical or very similar. The probe's detectable label provides a means for determining in a known manner whether hybridization has occurred.

In the use of the nucleotide segments as probes, the particular probe is labeled with any suitable label known to those skilled in the art, including radioactive and non-radioactive labels. Typical radioactive labels include ^{32}P, ^{35}S, or the like. Non-radioactive labels include, for example, ligands such as biotin or thyroxine, as well as enzymes such as hydrolases or peroxidases, or the various chemiluminescers such as luciferin, or fluorescent compounds like fluorescein and its derivatives. In addition, the probes can be made inherently fluorescent as described in International Application No. WO 93/16094.

Various degrees of stringency of hybridization can be employed. The more stringent the conditions, the greater the complementarity that is required for duplex formation. Stringency can be controlled by temperature, probe concentration, probe length, ionic strength, time, and the like. Preferably, hybridization is conducted under moderate to high stringency conditions by techniques well known in the art, as described, for example, in Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY., pp. 169-170.

As used herein "moderate to high stringency" conditions for hybridization refers to conditions that achieve the same, or about the same, degree of specificity of hybridization as the conditions "as described herein." Examples of moderate to high stringency conditions are provided herein. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes was performed using standard methods (Maniatis *et al.*). In general, hybridization and subsequent washes were carried out under moderate to high stringency conditions that allowed for detection of target sequences with homology to sequences exemplified herein. For double-stranded DNA gene probes, hybridization was carried out overnight at 20-25° C below the melting temperature (Tm) of the DNA hybrid in 6X SSPE, 5X Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula from Beltz *et al.* (1983):

Tm = 81.5°C + 16.6 Log[Na+]+0.41(% G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1X SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20°C for 15 minutes in 0.2X SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization was carried out overnight at 10-20°C below the melting temperature (Tm) of the hybrid in 6X SSPE, 5X Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes was determined by the following formula from Suggs *et al.* (1981):

Tm (°C)=2(number T/A base pairs) +4(number G/C base pairs)

Washes were typically carried out as follows:

(1) Twice at room temperature for 15 minutes 1X SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1X SSPE, 0.1% SDS (moderate stringency wash).

In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment of greater than about 70 or so bases in length, the following conditions can be used:

Low:	1 or 2X SSPE, room temperature
Low:	1 or 2X SSPE, 42°C
Moderate:	0.2X or 1X SSPE, 65°C
High:	0.1X SSPE, 65°C.

Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, polynucleotide sequences of the subject invention include mutations (both single and multiple), deletions, and insertions in the described sequences, and combinations thereof, wherein said mutations, insertions, and deletions permit formation of stable hybrids with a target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a

given polynucleotide sequence using standard methods known in the art. Other methods may become known in the future.

The mutational, insertional, and deletional variants of the polypeptide sequences of the invention can be used in the same manner as the exemplified polynucleotide sequences so long as the variants have substantial sequence similarity with the original sequence. As used herein, substantial sequence similarity refers to the extent of nucleotide similarity that is sufficient to enable the variant polynucleotide to function in the same capacity as the original sequence. Preferably, this similarity is greater than 50%; more preferably, this similarity is greater than 75%; and most preferably, this similarity is greater than 90%. The degree of similarity needed for the variant to function in its intended capacity will depend upon the intended use of the sequence. It is well within the skill of a person trained in this art to make mutational, insertional, and deletional mutations that are designed to improve the function of the sequence or otherwise provide a methodological advantage.

In one embodiment, the genes of the subject invention have at least one of the following characteristics:

said gene is encoded by a nucleotide sequence which hybridizes under stringent conditions with a nucleotide sequence selected from the group consisting of: DNA which encodes SEQ ID NO: 1, DNA which encodes SEQ ID NO: 2, DNA which encodes SEQ ID NO: 3, DNA which encodes SEQ ID NO: 4, DNA which encodes SEQ ID NO: 5 or SEQ ID NO: 6, DNA which encodes SEQ ID NO: 7 or SEQ ID NO: 8, DNA which encodes SEQ ID NO: 9, DNA which encodes SEQ ID NO: 10, and DNA which encodes SEQ ID NO: 11.

The subject invention also includes polynucleotides that hybridize with other polynucleotides of the subject invention.

<u>PCR technology</u>. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (see U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki *et al.*, 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of

the target sequence. The primers are oriented with the 3′ ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5′ ends of the PCR primers. Since the extension product of each primer can serve as a template for the other primer, each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, which is isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes that can be used are known to those skilled in the art.

The polynucleotide sequences of the subject invention (and portions thereof such as conserved regions and portions that serve to distinguish these sequences from previously-known sequences) can be used as, and/or used in the design of, primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5′ end) of the exemplified ppolynucleotides can be used in this manner. Mutations, insertions and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

Full length genes may be cloned utilizing partial nucleotide sequence and various methods known in the art. Gobinda *et al.* (1993; *PCR Methods Applic* 2:318-22) disclose "restriction-site PCR" as a direct method which uses universal primers to retrieve unknown sequence adjacent to a known locus. First, genomic DNA is amplified in the presence of primer to linker and a primer specific to the known region. The amplified sequences are subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR can be used to acquire unknown sequences starting with primers based on a known region (Triglia T. *et al.* (1988) *Nucleic Acids Res* 16:8186). The method uses

several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template. Divergent primers are designed from the known region. The multiple rounds of restriction enzyme digestions and ligations that are necessary prior to PCR make the procedure slow and expensive (Gobinda *et al.* [1993] *supra*).

Capture PCR (Lagerstrom M. *et al.* (1991) *PCR Methods Applic* 1:111-19) is a method for PCR amplification of DNA fragments adjacent to a known sequence in eucaryotic and YAC DNA. As noted by Gobinda *et al.* (1993,*supra*), capture PCR also requires multiple restriction enzyme digestions and ligations to place an engineered double-stranded sequence into an unknown portion of the DNA molecule before PCR. Although the restriction and ligation reactions are carried out simultaneously, the requirements for extension, immobilization and two rounds of PCR and purification prior to sequencing render the method cumbersome and time consuming.

Parker J. D. *et al.* (*Nucleic Acids Res* [1991] 19:3055-60), teach walking PCR, a method for targeted gene walking which permits retrieval of unknown sequences. PromoterFinder™ is a kit available from Clontech Laboratories, Inc. (Palo Alto, CA) which uses PCR and primers derived from p53 to walk in genomic DNA. Nested primers and special PromoterFinder™ libraries are used to detect upstream sequences such as promoters and regulatory elements. This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

A new PCR method replaces methods which use labeled probes to screen plasmid libraries and allow one researcher to process only about 3-5 genes in 14-40 days. In the first step, which can be performed in about two days, any two of a plurality of primers are designed and synthesized based on a known partial sequence. In step 2, which takes about six to eight hours, the sequence is extended by PCR amplification of a selected library. Steps 3 and 4, which take about one day, are purification of the amplified cDNA and its ligation into an appropriate vector. Step 5, which takes about one day, involves transforming and growing up host bacteria. In step 6, which takes approximately five hours, PCR is used to

screen bacterial clones for extended sequence. The final steps, which take about one day, involve the preparation and sequencing of selected clones.

If the full length cDNA has not been obtained, the entire procedure is repeated using either the original library or some other preferred library. The preferred library may be one that has been size-selected to include only larger cDNAs or may consist of single or combined commercially available libraries, *e.g.*, from Clontech Laboratories, Inc. (Palo Alto, CA). The cDNA library may have been prepared with oligo (dT) or random priming. Random primed libraries are preferred in that they will contain more sequences which contain 5' ends of genes. A randomly primed library may be particularly useful if an oligo (dT) library does not yield a complete gene. It must be noted that the larger and more complex the protein, the less likely it is that the complete gene will be found in a single plasmid.

CLONTECH PCR-Select™ cDNA Subtraction (Clontech Laboratories, Inc., Palo Alto, CA) is yet another means by which differentially expressed genes may be isolated. The procedure allows for the isolation of transcripts present in one mRNA population which is absent, or found in reduced numbers, in a second population of mRNA. Rare transcripts may be enriched 1000-fold.

A new method for analyzing either the size or the nucleotide sequence of PCR products is capillary electrophoresis. Systems for rapid sequencing are available from Perkin Elmer (Foster City CA), Beckman Instruments (Fullerton, CA), and other companies. Capillary sequencing employs flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled devise camera. Output/light intensity is converted to electrical signal using appropriate software (eg. Genotyper™ and Sequence Navigators™ from Perkin Elmer) and the entire process from loading of samples to computer analysis and electronic data display is computer controlled. Capillary electrophoresis provides greater resolution and is many times faster than standard gel based procedures. It is particularly suited to the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample. The reproducible sequencing of up to 350 bp of M13

phage DNA in 30 min has been reported (Ruiz-Martinez M. C. *et al.* [1993] *Anal Chem* 65:2851-8).

Polynucleotides and proteins. Polynucleotides of the subject invention can be defined according to several parameters. One characteristic is the biological activity of the protein products as identified herein. The proteins and genes of the subject invention can be further defined by their amino acid and nucleotide sequences. The sequences of the molecules can be defined in terms of homology to certain exemplified sequences as well as in terms of the ability to hybridize with, or be amplified by, certain exemplified probes and primers. Additional primers and probes can readily be constructed by those skilled in the art such that alternate polynucleotide sequences encoding the same amino acid sequences can be used to identify and/or characterize additional genes. The proteins of the subject invention can also be identified based on their immunoreactivity with certain antibodies.

The polynucleotides and proteins or polypeptides of the subject invention include portions, fragments, variants, and mutants of the full-length sequences as well as fusions and chimerics, so long as the encoded protein retains the characteristic biological activity of the proteins identified herein. As used herein, the terms "variants" or "variations" of genes refer to nucleotide sequences that encode the same proteins or which encode equivalent proteins having equivalent biological activity. As used herein, the term "equivalent proteins" refers to proteins having the same or essentially the same biological activity as the exemplified proteins.

Variations of genes may be readily constructed using standard techniques such as site-directed mutagenesis and other methods of making point mutations and by DNA shuffling, for example. In addition, gene and protein fragments can be made using commercially available exonucleases, endonucleases, and proteases according to standard procedures. For example, enzymes such as *Bal*31 can be used to systematically cut off nucleotides from the ends of genes. Also, genes that encode fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins. Of course, molecular techniques for cloning polynucleotides and producing gene constructs of interest are also well known in the art. *In vitro* evaluation

techniques, such as MAXYGEN's "Molecular Breeding" can also be applied to practice the subject invention.

Because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences encoded by the polynucleotide sequences disclosed herein. It is well within the skill of a person trained in the art to create these alternative DNA sequences encoding proteins having the same, or essentially the same, amino acid sequence. These variant DNA sequences are within the scope of the subject invention. As used herein, reference to "essentially the same" sequence refers to sequences that have amino acid substitutions, deletions, additions, or insertions that do not materially affect biological activity. Fragments retaining the characteristic biological activity are also included in this definition.

A further method for identifying genes and polynucleotides (and the proteins encoded thereby) of the subject invention is through the use of oligonucleotide probes. Probes provide a rapid method for identifying genes of the subject invention. The nucleotide segments that are used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures.

The subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalent proteins) having the same or similar biological activity of proteins encoded by the exemplified polynucleotides. Equivalent proteins will have amino acid similarity with an exemplified protein (or peptide). The amino acid identity will typically be greater than 60%. Preferably, the amino acid identity will be greater than 75%. More preferably, the amino acid identity will be greater than 80%, and even more preferably greater than 90%. Most preferably, amino acid identity will be greater than 95%. (Likewise, the polynucleotides that encode the subject polypeptides will also have corresponding identities in these preferred ranges.) These identities are as determined using standard alignment techniques for determining amino acid identity. The amino acid identity/similarity/ homology will be highest in critical regions of the protein including those regions that account for biological activity or that are involved in the determination of three-dimensional configuration that is ultimately responsible for the biological activity. In this

regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions which are not critical to activity or are conservative amino acid substitutions which do not affect the three-dimensional configuration of the molecule. For example, amino acids may be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound.

Table 4 provides a listing of examples of amino acids belonging to each class.

Table 4.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the biological activity of the polypeptide.

An "isolated" or "substantially pure" nucleic acid molecule or polynucleotide is a polynucleotide that is substantially separated from other polynucleotide sequences which naturally accompany a nucleic acid molecule. The term embraces a polynucleotide sequence which was removed from its naturally occurring environment by the hand of man. This includes recombinant or cloned DNA isolates, chemically synthesized analogues and analogues biologically synthesized by heterologous systems. An "isolated" or "purified" protein or polypeptide, likewise, is a one removed from its naturally occurring environment.

Materials and Methods

Cells and virus.

AmEPV (Hall, R. L. and R.W. Moyer [1991] *supra*) was replicated in IPLB-LD-652 cells (Goodwin, R. H., J. R. Adams and M. Shapiro [1990] "Replication of the entomopoxvirus from *Amsacta moorei* in serum-free cultures of a gypsy moth cell line" *J. Invertebr. Pathol.* 56:190-205) which were maintained at 28°C in a 1:1 mixed medium (TE medium) of TC-100 media (Gibco, Gaithersburg, MD) and EX-CELL 401 media (JRH Biosciences, Lenexa, KS), supplemented with 10% fetal bovine serum. A TK negative cell line designated C11.3 was selected by a process of adaption of TK(+) LD652 cell to increasing levels, 10 μg/ml every 5 weeks, of 5-bromo-2′-deoxyuridine (BudR) over one year up to 100 μg/ml BudR and maintained in TE medium containing BudR (100 μg/ml). 293 cells were grown in DMEM medium supplemented with 5% fatal bovine serum.

Plasmid construction and preparation of AmEPV recombinant.

pTR-UF5 (see Figure 1, provided by the Vector Core, Gene Therapy Center, University of Florida) contains GFP and NeoR genes under control CMV promoter and herpes virus TK promoter respectively and flanked by ITR sequences of AAV. The Pst I fragment which contains GFP and NeoR markers was inserted into Pst I site of pTKDU (Li, Y., R.L. Hall, S.L. Yuan, R.W. Moyer [1998] "High level expression of *Amsacta moorei* entomopoxvirus *Spheroidin* depends on sequences within the gene" *J. Gen. Virol.* 79:613-622) to produce pTKUF5. AmEPV recombinant with an insert in the TK gene was obtained as described previously (Li *et al.* [1998] *supra*).

Viral genomic DNA preparation

Growth and maintenance protocols for IPLB-LD-652 cells and AmEPV are described in detail in Bawden *et al.*, 2000. DNA was obtained from amplification of a single wtAmEPV plaque (Bawden, A. L., Li, Y., Maggard, K., and Moyer, R. W. [2000] Entomopoxvirus Vectors. *In* "Viral Vectors: Basic Science and Gene Therapy," A. Cid-Arregui, Ed., Eaton Publishing, Natick, MA (In Press)). Thirty 150 cm^2 dishes containing

approximately 2.4×10^7 LD652 cells (Goodwin, R. H., Adams, J. R., and Shapiro, M. [1990] "Replication of the Entomopoxvirus from Amsacta moorei in Serum-Free Cultures of a Gypsy Moth Cell Line" *J. Invertebr. Path* 56:190-205) were infected at an MOI of 0.01 with wild type AmEPV and incubated at 28°C. Infections (cells and medium) were harvested 6 days post-infection and centrifuged at 500 x g for 15 minutes to remove cells. The supernatant was centrifuged at 40,000 x g for 30 minutes to pellet virus. The pellet was resuspended in dH_2O (100 µL for each initial 30 mL of supernatant). DNase free RNase was added to a final concentration of 50 µg/mL and incubated at 37 °C for 30 min. The sample and lysis buffer (100mM Tris pH 8.0, 10 mM EDTA, 54% sucrose, 2% SDS, 10mM β-mercaptoethanol) were brought to 50°C, and lysis buffer was added to the sample at a 1:1 ratio. Proteinase K was added to a final concentration of 0.6 mg/mL. The viral lysate was incubated overnight at 50°C. The lysate was extracted three times with 50:49:1 phenol:chloroform:isoamylalcohol, once with chloroform, and the DNA precipitated in 0.4 M $LiCl_2$, 95% ethanol.

*Tsp*509I partial digest library preparation

Ten micrograms of AmEPV DNA were digested with 5 units of *Tsp*509I. Two aliquots were removed at 3 and 6 minute time points and digestion stopped with 50 mM EDTA (final concentration). This method was repeated in triplicate for a total of 30 µg of digested DNA. Fragments of 2-3 kb and 4-5 kb were gel-purified separately with the Gene-Clean II kit (Bio 101, Vista, CA) and ligated into the *EcoRI* site of the PUC19 plasmid vector (Amersham Pharmacia Biotech UK Ltd., Chalfont, Buckinghamshire, England). The ligation mixture was transformed into DH5-α competent cells and plated onto LB agar plates containing 50 µg/mL ampicillin and 800 µg/plate each IPTG and X-gal (Horton, P. and Nakai, K. [1997] "Better prediction of protein cellular localization sites with the k nearest neighbors classifier" *Ismb.* 5:147-152). White colonies were isolated and grown overnight in 1 mL TB medium (Horton & Nakai [1997] *supra*) plus 50 µg/mL ampicillin.

Sequence determination

Plasmid DNA was prepared using the QIAgen BioRobot 9600 and the QIAprep 96 Turbo miniprep kit. Sequencing was performed with 200-500ng of plasmid DNA as template using a 0.25X concentration of ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (#4303153; Perkin-Elmer Applied Biosystems [ABI], Foster City, CA). Cycle sequencing was performed using a PTC-200 DNA Engine (MJ Research, Watertown, MA) (25 cycles: 1 degree per second to 96 degrees; 96 for 10 seconds; 1 degree per second to 60 degrees; 60 for 4 minutes). Dye terminator removal was on Multiscreen-HV plates (Millipore) with Sephadex G-50 superfine (Sigma, St. Louis, MO) in water. The reactions were electrophoresed on an ABI 377 sequencer, and the chromatograms were edited with Analysis version 1.2.1 (ABI) and assembled as follows.

Sequence Assembly and Analysis

Chromatograms were assembled into "contigs" using the Phred/Phrap/Consed software package (Horton, P. and Nakai, K. [1996] "A probabilistic classification system for predicting the cellular localization sites of proteins" *Ismb.* 4:109-115; Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. [1997] "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs" *Nucleic Acids Res.* 25:3389-3402; Bateman, A., Birney, E., Durbin, R., Eddy, S. R., Finn, R. D., and Sonnhammer, E. L. [1999] "Pfam 3.1: 1313 multiple alignments and profile HMMs match the majority of proteins" *Nucleic Acids Res.* 27:260-262). After assembling 3500 chromatograms into 6 contigs, Consed designed 43 finishing experiments. Custom oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA), and upon completion of the experiments, the assembly contained the entire unique region of the genome and one inverted terminal repeat (ITR). After further data manipulation using the programs phrapview and miropeats, the ITR regions on either end were delineated (Horton & Nakai [1996] *supra*; Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. [1990] "Basic local alignment search tool" *J. Mol. Biol.* 215:403-410). The consensus was sequenced to an average redundancy of 10X. The sequence was confirmed

by comparision to *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, and *Xho*I, restriction maps of AmEPV (Hall, R. L. and Hink, W. F. [1990] "Physical mapping and field inversion gel electrophoresis of *Amsacta moorei* entomopoxvirus DNA" *Arch. Virol.* 110:77-90). The sequence of AmEPV has been deposited into GenBank, nucleotide acession number AF250284. Methionine-initiated open reading frames were delineated using Vector NTI. Open reading frames that translated into proteins less than 60 amino acids were discarded from our analysis. Relevant homologies were determined by BLAST analysis (Parsons, J. D. [1995] "Miropeats: graphical DNA sequence comparisons" *Comput. Appl. Biosci.* 11:615-619; Ewing, B., Hillier, L., Wendl, M. C., and Green, P. [1998] "Base-calling of automated sequencer traces using phred. I. Accuracy assessment" *Genome Res.* 8:175-185), and additional domains found using the Pfam program (Gordon, D., Abajian, C., and Green, P. [1998] "Consed: a graphical tool for sequence finishing" *Genome Res.* 8:195-202). Default E (EXPECT) values of <0.01 were used to define homology to sequences in current databases. Transmembrane (TM), leucine zipper, and signal peptide (SP) domains were predicted by the Psort program (van Veen and Konings [1998] *supra*; Ewing, B. and Green, P. [1998] "Base-calling of automated sequencer traces using phred. II. Error probabilities" *Genome Res.* 8:186-194).

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

<u>Example 1 — Gene expression in cells infected with recombinant AmEPV</u>

293 cells (1×10^6) were placed in 6-well plate and infected with recombinant AmEPVpTKUF5 or AmEPVpTKespgfp (Li *et al.* [1997] *supra*) viruses at a multiplicity of five (5) virus particles/cell. As controls, cells were separately transfected with either the plasmid pTR-UF5 or pTKUF5 at a 5 μg/well plasmid DNA. Two days later, virus infected or plasmid transfected cells were transferred into 60 mm dishes, after 24 hr, neomycin resistant colonies were selected by adding G418 at the final concentration of 200 μg/ml. G418 containing medium was changed every 3-4 days.

For cells infected with recombinant AmEPV pTKespgfp, no neomycin resistant colony was observed, an expected result since this virus does not have NeoR gene. However, in cells infected with recombinant AmEPV pTKUF5 or transfected with plasmids pTR-UF5, G418 resistant colonies were observed. All colonies from cells transfected with either of the two plasmids were both G418 resistant and GFP positive. However, colonies from cells infected with recombinant pTKUF5 were initially only G418 resistant, and not GFP positive. G418 resistant colonies derived from the AmEPV recombinant also grew more slowly than those produced following plasmid transfection. Most likely, the explanation for these results is that GFP and NeoR gene copy number in AmEPV derived colonies is less than those transfected with plasmids. This explanation is likely to be true as we were able to show that the AmEPV derived colonies gradually become more and more resistant to G418 and soon, some GFP positive clusters of cells were observed which become more numerous and brighter. After several changes of medium, ultimately, all cells in the well were GFP positive.

<u>Example 2 — Stable integration of foreign DNA sequences into mammalian cells infected with recombinant AmEPV</u>

Genomic DNA was recovered from cell lines created by either infection with the virus AmEPVpTKUF5 or following transfection with a control plasmid pTR-UF5. Specifically, the recombinant AmEPVpTKUF5 was used to infect and subsequently select 293 (human kidney) cells at a multiplicity of 5 plaque forming units per cell, as described in Example 1. After growing the isolated cell lines reliably for multiple generations, DNA was isolated and digested with *Hind*III before electrophoresis and blotted with a random labeled probe containing the *gfp* and neo genes which are contained within the ITR regions of pTR-UF5. As shown in Figure 2, lane P contains genomic DNA from 293 cells and pTR-UF5 plasmid, showing excision of the cassette from the plasmid upon digestion. A control (not shown) of 293 cells alone did not produce any endogenous cross-reacting bands. As seen in Figure 2, the host chromosomal site in the 293 genome of integration is random, as evidenced by the different sized bands resulting from *Hind*III digestion. In some cell lines,

the event can be seen to have occurred more than once (multiple copies have integrated). Directional integration into the long arm of chromosome 19 would be expected if the *rep* gene of AAV were simultaneously expressed. This experimental data proves delivery and stable integration of foreign DNA sequences by AmEPV.

<u>Example 3 — Growth and amplification of AmEPV</u>

AmEPV productively infects *Lymantria dispar*-derived IPLB-LD-652 (LD) cells (Goodwin *et al.,* 1978). LD cells were maintained at 28°C in a 1:1 ratio of TC-100 medium (Gibco, Gaithersburg, MD) to EX-CELL 401 medium (JRH Biosciences, Lenexa, KN) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (1:1 TE). To amplify virus, cells grown in 150 mm dishes are inoculated at a multiplicity of infection (m.o.i.) of 0.01 in sufficient media (5 ml) to cover the surface of the tissue culture vessel. The cells should be no more than 70% confluent. After adsorption at 28°C for 2 hr, 25 ml medium is added and the infections are incubated for 4-6 days at 28°C. The infection is considered complete when most cells become occlusion body positive as seen by light microscopy, i.e. when refractile occlusion bodies can be seen. For recombinant viruses in which *lacZ* has been inserted into either the TK or the spheroidin locus, infection is monitored by *in situ* staining of infected cells with 1 mg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal), 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4, pH 7.4). This stain allows β-galactosidase producing infected cells to be visualized by the appearance of blue color. Though numbers can vary, an AmEPV infection at an m.o.i. of 0.01 generates a net yield of approximately 1.5 plaque forming units (PFU) based on the total number of cells and results in a 100-fold increase in PFU. Titers are routinely between 10^5 and 10^6 PFU/ml. Cells infected at a higher m.o.i. can generate higher yields/cell (~10) but the net increase in virus over input is lower.

Example 4 — Partial purification and concentration of virus

Some experiments necessitate higher concentrations of virus. The procedure that follows typically renders semi-pure virus stocks at titers > 10^8 PFU/ml. AmEPV infected cells are harvested by scraping and centrifuged at 700 x *g* for 15 min to remove cells. The supernatant is then subjected to ultracentrifugation at 70,000 *x g* for 2 hr to pellet the extracellular virus. The virus is resuspended in an appropriate amount of PBS (typically 100 μl per 40 ml of supernatant) and the titer is determined by plaque assay. Total yield is typically 50% of input virus.

Example 5 – Plaque Assay

Virus to be titered is subjected to 10-fold serial dilutions in 1:1 TE medium. LD-652 cells are plated at 70% confluency in 6-well dishes, each having a 34.6 mm diameter (roughly 1.4 x 10^6 cells per well). Once the cells have adhered, the medium is removed and 0.5 ml of diluted virus is added to the wells. After adsorption at 28°C for 2 hr, the inoculum is removed and 2.5 ml of overlay is added to each well. The overlay is a 2:1 ratio of 1.33X TC-100 medium (containing 14% fetal calf serum) and 4% sterile low melting point agarose, equilibrated to 42°C and mixed just prior to addition to the monolayer.

For a spheroidin-positive virus, visible plaques appear and are counted one week post infection (Figure 3). Spheroidin-negative virus plaques are much more difficult to visualize, hence most spheroidin negative viruses have been engineered to contain *lacZ*. Such viruses can be readily visualized by staining with Xgal as follows: a liquid overlay of 400 μg Xgal in 50 μl total solution is spread over the agarose in each well of the plaque assay 3-4 days post-infection. Plaques appear as blue patches of infected cells and are counted one week post-infection.

Comet-like plaques of wtAmEPV, in the absence of an agarose overlay can be more rapidly visualized by immunostaining (Winter, J., R.L. Hall, and R.W. Moyer [1995] "The effect of inhibitors on the growth of the entomopoxvirus from *Amsacta moorei* in *Lymantria dispar* (gypsy moth) cells" *Virology* 211:462-473). Plaque assays are prepared as above, except that a liquid overlay of medium replaces the agarose overlay. Infected cell

monolayers are air dried three days postinfection and fixed in acid alcohol (95% EtOH, 5% glacial acetic acid) for 30 minutes. After equilibration with TBS (0.02 M Tris pH 7.4,0.15 M NaCl) the cells are incubated in TBS-Block (0.5% w/v blocking reagent in TBS [Boehringer Mannheim, Germany]) for 1 hr at room temperature to prevent nonspecific antibody binding. The primary antibody (rabbit anti-AmEPV occlusion body antiserum) (Hall *et al.* [1996] *supra*) or secondary antibody (goat anti-rabbit conjugated to alkaline phosphatase; Fisher, Atlanta, GA) are both diluted in TBS-Block. Antibody reactions and color development are performed as previously described (Harlow. E., and D. Lane [1998] *Antibodies*- A Laboratory Manual, E. Harlow and D. Lane, Eds., pp. 635-657. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Example 6 — Isolation of AmEPV genomic DNA

LD-652 cells (typically 10^9 cells, thirty 150 mm dishes) are infected with AmEPV at an m.o.i. of 0.01. The infections (cells and medium) are harvested by scraping and centrifuged at 700 x *g* for 15 min. to remove cells. The supernatant is centrifuged at 39,000 x *g* for 30 min. to pellet extracellular virus. The viral pellet is resuspended in deionized water (100 µl for 40 ml of supernatant). DNAase free RNAase is added to the resuspended viral pellet at a final concentration of 50 µg/ml and incubated at 37°C for 30 min. The virus sample is then heated to 50°C, and an equal volume of lysis buffer (100 mM Tris pH 8.0, 10 mM EDTA, 54% sucrose, 2% SDS, 10 mM β-mercaptoethanol) is added to the sample. Proteinase K is then added to a final concentration of 0.6 mg/ml, and the viral lysate is incubated overnight at 50°C. The lysate is extracted three times with 50:49:1 phenol:chloroform:isoamylalcohol and once with chloroform, and the DNA is precipitated in 0.4 M LiCl, 95% ethanol. This procedure typically yields 2 µg of genomic AmEPV DNA per 10^7 infected cells.

Example 7 — Shuttle vector plasmid construction

Following the sequence determination of two non-essential genes, thymidine kinase (TK) and spheroidin (Gruidl *et al.* [1992] *supra*; Hall and Moyer [1991] *supra*), we were able to create shuttle vector plasmids for the generation of recombinant AmEPV viruses. The shuttle vectors are described below.

A. The TK insertion site shuttle vector: Oligonucleotide primers were used to PCR amplify a 748-bp fragment of downstream TK flanking sequence from plasmid pMEGTK-1 (Gruidl *et al.* [1992] *supra*). Another set of oligonucleotide primers was used to PCR amplify a 663-bp fragment of TK upstream flanking sequence from pMEGTK-1. These two fragments were separately inserted into pBluescript I SK(+) to produce pDUTK (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562). Foreign genes were then cloned within the TK flanks to generate shuttle vectors for the generation of recombinants (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562).

B. The spheroidin insertion site shuttle vector: Oligonucleotides were used to PCR amplify a 1046-bp fragment of upstream spheroidin flanking sequence from plasmid pRH512 (Hall and Moyer [1991] *supra*). In addition to 1023-bp of sequence upstream of the spheroidin gene, this fragment contained the starting ATG of the spheroidin coding sequence and twenty base pairs following the ATG. Another set of oligonucleotide primers was used to amplify a 998-bp fragment of downstream spheroidin flanking sequence from pRH512. These two fragments were separately inserted into pBluescript I SK(+) to produce pDU20 (Hall, R. L., Li, Y., Feller, J. A., and Moyer, R. W. [1996] "The *Amsacta moorei* entomopoxvirus spheroidin gene is improperly transcribed in vertebrate poxviruses" *Virology* 224:427-436). Subsequent constructs were cloned within the spheroidin flanks to generate various shuttle vectors for the generation of recombinants (Hall *et al.* [1996] *Virology* 224:427-436).

C. AmEPV early promoter constructs: Promoters for early poxvirus genes are active prior to viral DNA replication. We have utilized two early EPV promoters in our constructs.

The first, an AmEPV early strong promoter (*esp*) was derived from a strongly expressed 42 kDa early protein (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562). The second promoter was derived from the early expressed fusolin (*fus*) gene as described (Gauthier *et al.* [1995] *supra*). These promoters have been used to regulate reporter genes (*lacZ, gfp*). The appropriately regulated genes are then placed within shuttle vectors and transfected into infected cells to produce recombinant viruses. The shuttle vector pTK-fus*lacZ* was constructed by PCR amplification of the MmEPV fusolin early promoter from pHF51 and insertion into pDUTK; *lacZ* was subcloned from pMC1871 (Pharmacia Biotech, Inc., Piscataway, N.J.) as described (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562). The shuttle vector pTK-esp*lacZ* (pTK-42k*lacZ*) was constructed by cloning the PCR amplified *esp* promoter into pTK-fus*lacZ* after excision of the fusolin promoter (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562). To construct pTK-esp*gfp*, a green fluorescent protein gene (*gfp*) was PCR-amplified from the pTR-UF5 plasmid (Vector Core, University of Florida) (18) and cloned into pTK-esp*lacZ* replacing the *esp*lacZ cassette as described in (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562).

D. <u>AmEPV late promoter constructs</u>: We have used the spheroidin (*sph*) promoter as an example of an AmEPV strong late promoter. This promoter has two rather unexpected properties: (1) the *sph* promoter appears to be insect cell specific and functions very poorly in vertebrate cells (Hall *et al.* [1996] *supra*), and (2) unlike previously described late poxvirus promoters, we have found in insect cells that expression is further enhanced by including the 20 bp downstream of the TAAATG promoter in the reporter gene constructs (23). pDU20*lacZ* was created by insertion of *lacZ* (from plasmid pMC1871, Pharmacia Biotech, Inc., Piscataway, NJ) into the *Bam*HI site of pDU20. The final reporter contains 1046 bp of potential spheroidin promoter sequence plus 20 bp of additional downstream

spheroidin coding sequence following the TAAATG sequence before fusion to lacZ (Hall *et al.* [1996] *supra*). pDU2*lacZ* was constructed using the same strategy as that for pDU20*lacZ*, except that only 2 bp of spheroidin coding sequence follows the translation-starting TAAATG before fusion to *lacZ* (Hall *et al.* [1996] *supra*, Li, Y., R.L. Hall, S. Yuan, and R.W. Moyer [1998] "High-level expression of *Amsacta moorei* entomopoxvirus *spheroidin* depends on sequences within the gene" *J. Gen. Virol.* 79:613-622). We have also constructed and used the cowpoxvirus late ATI gene promoter to drive *lacZ* which functions well in both insect and vertebrate cells (Li, Y., R.L. Hall, S. Yuan, and R.W. Moyer [1998] "High-level expression of *Amsacta moorei* entomopoxvirus *spheroidin* depends on sequences within the gene" *J. Gen. Virol.* 79:613-622).

E. Construct driven by Pol II specific promoters: An AmEPV construct containing reporter genes driven by Pol II rather than poxvirus promoters has also been prepared based on the plasmid pTR-UF5 (Vector Core, University of Florida) (Klein, R. L., E. M. Meyer, A. L. Peel, S. Zolotukhin, C. Meyers, N. Muzyczka, and M.A. King [1998] "Neuron-specific transduction in the rat septohippocampal or nigrostriatal pathway by recombinant adeno-associated virus vectors" *Experimental Neurology* 150:183-194) which is a plasmid containing two mammalian reporter genes: (1) a gene coding for neomycin resistance (*neoR*) driven by the herpes simplex virus TK promoter and (2) *gfp* driven by the immediate early promoter/enhancer from cytomegalovirus (CMV). The dual gene cassette is flanked by the AAV-inverted terminal repeats (ITRs). pTR-UF5 was digested with *Sal*I to remove two *Pst*I sites then religated to form pTRUF5)*Sal*I. This construct was then digested with *Pst*I, and the fragment containing the two reporter genes was inserted into the *Pst*I site of pDUTK to produce pTKUF5)*SalI.* This construct was then digested with *Sal*I and the previously removed *Sal*I fragment was reinserted into the construct to produce pTKUF5 (Figure 1).

Example 8 — Generation and Selection of recombinant AmEPV

Neither the spheroidin gene nor the thymidine kinase gene is required for propagation of AmEPV in cell culture (Palmer, C.P., D.P. Miller, S.A. Marlow, L.E. Wilson, A.M. Lawrie, and L.A. King [1995] "Genetic modification of an entomopoxvirus: deletion of the

spheroidin gene does not affect virus replication *in vitro*" *J. Gen. Virol.* 76:15-23; R.W. Moyer, Li, Y. and Bawden, A., unpublished results), providing sites for insertion of foreign genes by homologous recombination. Following transfection of AmEPV infected LD-652 cells, all foreign genes are inserted into either the TK or the spheroidin locus (Hall *et al.* [1996] *supra*).

A. Transfection of infected cells: LD-652 cells (1.4 x 10^6 cells, 70% confluent in a 34.6 mm dish) are infected with AmEPV at an m.o.i. of 5 PFU per cell in a volume of 1 ml. Two hours post-infection, the inoculum is aspirated and 1 ml of transfection mix + DNA is added.

Transfection mix + DNA is prepared by separately combining 20 μl Lipofectin (Gibco, Gaithersburg, MD) and 80 μl 1:1 TE media without FBS, and 5 μg of shuttle vector plasmid DNA in a volume of 100 μl of the same media. The mixture is incubated at room temperature for 15 min. The concentrated transfection mix is then diluted by addition of 800 μl of 1:1 TE without FBS and added to cells. After 6 hr, the transfection mix is removed and replaced with 2 ml of 1:1 TE with 10% FBS. Three days post-infection the supernatant is harvested by centrifugation at 200 x *g*.

B. Selection of AmEPV recombinants: For selection of recombinants inserted into the TK gene, a TK(-) cell line, C11.3, was derived by serial passage of LD-652 cells in increasing concentrations of 5-bromo-2'-deoxyuridine (BudR) (10 μg/ml increasing increments of BudR at intervals of five weeks over one year). C11.3 cells are maintained at in 1:1 TE medium supplemented with 100 μg/ml BudR (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562). Supernatants harvested from the infection/transfection described above are plaqued on C11.3 cells in the presence of 100 μg/ml BudR. Only virus lacking a functional TK gene, i.e. recombinant virus, will grow in the presence of BudR. For selection of recombinants where insertion is within the spheroidin gene (where *lacZ* has been incorporated into the shuttle plasmid) recombinant virus plaques are identified following staining with Xgal as described above or by the selection of non-refractile plaques.

C. Purification of recombinants: Dilutions at 10^{-2} to 10^{-4} of the infected cell supernatant are assayed by repeated plaquing (described above). Individual plaques are isolated and diluted in 1 ml 1:1 TE medium plus 10% FBS. Isolates are replaqued at dilutions at 10^{-1} to 10^{-3}. The plaque purification is repeated 3-4 times prior to plaque expansion for the propagation of larger stocks. These viruses can be stored at 4 oC for 2-3 months or at -80^{O}C for long-term storage.

Example 9 – Foreign gene expression in permissive insect cells

In our hands, we find the level of foreign gene expression within infected insect cells when driven by either of two late promoters (the cowpox virus ATI or spheroidin +20 promoters) equals or exceeds that of vaccinia or baculoviruses on a per/cell basis. The technology needs no elaboration, as once suitable recombinant viruses are constructed, only appropriate infection of insect cells is necessary.

Example 10 — Transient expression in vertebrate cells

The normal host range of AmEPV is limited to Lepidoptera (butterflies), and early experiments attempting to infect vertebrate cells with AmEPV indicated no obvious deleterious effects on the cells. Given the general promiscuity of poxviruses in the binding and entering of cells and the similarity of the AmEPV life cycle to that of vaccinia, we had reason to believe AmEPV would infect and enter vertebrate as well as insect cells. AmEPV recombinants were constructed carrying the *lacZ* reporter gene regulated by either of two early AmEPV promoters, the late spheroidin promoter or the ATI promoter from cowpox (TK-fus*lacZ*, TK-esp*lacZ*, SPH(20)*lacZ* and TK-ATI*lacZ*, described above). When mammalian CV-1 cells were infected with the recombinant viruses at an m.o.i. of 10 PFU/cell, those cells infected with AmEPV recombinants where *lacZ* was regulated by either the MmEPV fusolin or the AmEPV 42kD protein early promoters, expressed β-galactosidase (Figure 3, panels D and E) (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562). No expression was observed for the recombinants where *lacZ* was under control of either of the two late promoters (Figure 3, panels B and C). Thus, AmEPV enters

vertebrate cells and undergoes at least a partial uncoating which allows early, pre-replicative viral genes to be expressed. The infection is blocked thereafter and neither viral DNA replication nor late protein synthesis is observed. Early expression results from the fact that AmEPV, like other poxviruses, packages the enzymes necessary for early gene transcription within the virion. However, if vertebrate cells are co-infected with both VV and AmEPV, late promoters within AmEPV are rescued and activated suggesting that vaccinia can provide factors *in trans* which are needed for the infection to progress and activate the late promoters.

While determining the basis of host range restriction is difficult, the cytoplasmic nature of AmEPV coupled with a virus encoded transcription and replication machinery offers major advantages for vector design. We can be fairly certain that late genes are *not* transcribed because of the lack of β-galactosidase expression from late promoters in vertebrate cells and because DNA synthesis, a requirement for late mRNA synthesis does not occur. It is quite possible that incomplete uncoating of the virus leads to the block in gene expression.

Normally, uncoating of poxviruses occurs in two discrete steps. Upon entry into cells, virions are sufficiently permeabilized to allow early gene transcription from the viral core. Early proteins allow the complete uncoating of the core to allow transcription of the later classes of genes following interaction of newly synthesized DNA with intermediate and late transcription factors. The uncoating of vertebrate poxviruses has been thoroughly studied, and uncoating intermediates have been identified through differential centrifugation of cellular extracts infected with labeled virus. A viral activity specifically required for the second stage of uncoating has been identified. By analogy with vaccinia, AmEPV might be expected to encode a similar uncoating factor. If so, then one would not necessarily expect a cell-line specific block in uncoating unless this uncoating protein acts in conjunction with cellular components. Should we find that the particle *is* uncoated, then AmEPV fails to express other genes in vertebrate cells required for the infection to continue. Host range restriction of another insect virus family, the *Baculoviridae*, has received considerable attention. For baculoviruses, blockage may occur at many stages during the activation of late or very late genes after viral DNA enters the nucleus (Carbonell, L.F., M.J. Klowden, and

L.K. Miller [1985] "Baculovirus-mediated expression of bacterial genes in dipteran and mammalian cells" *J. Virol.* 56:153-160; Carbonell, L.F. and L.K. Miller [1987] "Baculovirus interaction with nontarget organisms: a virus-borne reporter gene is not expressed in two mammalian cell lines" *Appl. Environ. Microbiol.* 53:1412-1417; Morris, T.D. and L.K. Miller [1992] "Promoter influence on baculovirus-mediated gene expression in permissive and nonpermissive insect cell lines" *J. Virol.* 66:7397-7405; Morris, T.D. and L.K. Miller [1993] "Characterization of productive and non-productive AcMNPV infection in selected insect cell lines" *Virology* 197:339-348). The questions related to host range specificity are outside the scope of this review, but the limit of expression to those genes under the control of early promoters following infection of vertebrate cells is a key property of the virus which ultimately makes it a potential candidate as a vector for transient expression in vertebrate cells.

The most novel feature of this system is the survival and continued growth of the infected vertebrate cells. We immediately noticed that there were no observable cytopathic effects in the infected CV-1 cells. Survival and growth of the infected cells was shown following infection with recombinant AmEPV TK-esp*gfp*. Initially, individual fluorescent cells resulted, which over a period of two to three days, divided to form fluorescent microclusters of cells (Figure 4). There is no other known precedent of cells surviving a poxvirus infection. The fact that mammalian cells survive suggests that AmEPV offers the potential for a highly efficient, nontoxic method of foreign gene delivery into vertebrate cells for transient expression of foreign genes, after which the cells continue to grow unabated. While initial observations were made using CV-1 cells, these results have been extended to many other cell lines. In general, lymphocytic cells are more resistant to infection (Li, Y., Hall, R. L., and Moyer, R. W. [1997] "Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses" *J. Virol.* 71:9557-9562).

Example 11— AmEPV mediated gene expression in the mouse

To examine the feasibility of AmEPV to deliver and express foreign genes *in vivo*, we examined the effects of injection of TK-esp*lacZ* and SPH(20)*lacZ* into mouse muscle.

Approximately 2 x 106 PFU (100 µl) of each virus was injected into the muscle of the hind leg of two separate mice; as an additional control, we also injected 100 µl PBS into a third mouse. Two days post infection, the mice were sacrificed and the muscle was excised into small pieces, fixed in a 3% formaldehyde solution for 30 min, and stained with X-gal. Extensive β-galactosidase expression occurred in the muscle from the TK-esp*lacZ* infected mouse (Figure 5). No expression was seen in either the SPH(20)*lacZ* or the PBS control. Thus, consistent with our observations of infected mammalian cells in culture, AmEPV can also enter cells *in vivo* and allow early, but not late expression of a reporter gene.

Example 12 — The control of AmEPV induced inflammation

One concern with complex viral vectors is the potential for the unintended induction of inflammatory and immunological responses following administration. In studies with adenovirus, inflammation and immunogenicity to the virus and to virus-infected cells has limited transgene expression and the utility of this approach to treat chronic illnesses. Inflammation is initially characterized by perivascular and peribronchiolar inflammatory cell infiltration. Neutrophils and later macrophages and lymphocytes frequent the site of the infected area. Specific cytokines can also be measured as an index of the inflammatory response (Ginsberg, H.S., L.L. Moldawer, P.B. Sehgal, M. Redington, P.L. Kilian, R.M. Chanock, and G.A. Prince [1991] "A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia" *Proc. Natl. Acad. Sci. U.S.A.* 88:1651-1655; Noah, T.L., I.A. Wortman, P.C. Hu, M.W. Leigh, and R.C. Boucher [1996] "Cytokine production by cultured human bronchial epithelial cells infected with a replication-deficient adenoviral gene transfer vector or wild-type adenovirus type 5" *Am. J. Respir. Cell Mol. Biol.* 14:417-424). The early response to adenovirus infection consists of diffuse cellular infiltration of peribronchiolar and alveolar regions associated with the appearance of several classes of pro-inflammatory cytokines (Ginsberg *et al.* [1991] *supra*; Noah *et al.* [1996] *supra*). These include TNF-a, IL-1, IL-6, and IL-8 (KC/GRO in the mouse). There is considerable experimental evidence from rodents demonstrating that these classes of cytokines, and in

particular TNF-a and IL-8 (or KC/GRO), play central roles in the recruitment and activation of inflammatory cell populations in the lung.

It is likely with a virus as complex as AmEPV that unintended inflammation will result when the virus is introduced *in vivo*. However, vertebrate poxviruses may serve as a source of genes to provide a solution to this problem. There have been a variety of vertebrate poxvirus-encoded secreted virokines and viroceptors described including IFN-"/β, IFN-(, TNF and IL-1, and chemokine receptors (Barry, M. and G. McFadden [1997] "Virus encoded cytokines and cytokine receptors" *Parasitology* 115:S89-100; Smith, G.L., J.A. Symons, A. Khanna, A. Vanderplasschen, and Alcami, A. [1997] "Vaccinia virus immune evasion" *Immunol. Rev.* 159:137-154:137-154; Turner, P.C. and R.W. Moyer [1998] "Control of apoptosis by poxviruses" *Seminars in Virology* 8:453-469). Should inflammation be observed, cloning any or all of these genes into AmEPV to control any AmEPV induced inflammation is quite plausible.

Example 13 — The use of AmEPV to stably transform mammalian cells

A. Considerations in the design of AmEPV vectors for stable transformation: Poxviruses are cytoplasmic and therefore poxvirus promoters are recognized only by the poxvirus encoded RNA polymerase and not by RNA polymerase II of the host cell. Since we wished to demonstrate the ability of AmEPV to mediate stable transformation of mammalian cells, we constructed a recombinant AmEPV containing a cassette in which *gfp* and a gene conferring neomycin resistance were cloned under the control of promoters recognized by the *cellular* (not poxvirus) RNA polymerase (Figure 6). The promoters chosen were the CMV immediate early and herpesvirus TK gene promoters respectively, and the cassette was flanked by the inverted terminal repeat (ITR) sequences of AAV.

Although the exact mechanism of AAV site specific integration is unknown, two required viral components have been identified. These are the inverted terminal repeat sequences of AAV DNA (ITRs) and the Rep 78/68 proteins. The ITRs comprise two 145-nucleotide elements located at either end of the AAV genome. ITR sequences enclosing marker genes have been shown to allow a lower level of random genome integration when

compared to the levels of specific integration observed when genes encoding the Rep 78/68 proteins are also included in constructs.

B. Selection of stable recombinants: When vertebrate cells are transfected with plasmid pTK-UF5, and selected with G418 (250mg/ml) 24 hours later, colonies of resistant, fluorescent vertebrate cells are observed after about ten days of selection. In contrast, colonies from cells infected with AmEPVpTK-UF5 at an m.o.i. of five were G418 resistant, but not initially GFP positive. Approximately 20 G418-resistant clones can be isolated following infection of one million cells (one 35 mm dish). The efficiency of transformation is only 5-10 fold less than that for optimized plasmid mediated transfections. These AmEPV-derived colonies were observed to initially grow more slowly in the presence of G-418 than those produced from a plasmid transfection. Most likely, the explanation for these results is that the GFP and NeoR gene copy number in AmEPV-derived colonies is less than that for clones derived from cells transfected with the pTKUF5 plasmid. This explanation is further supported by the observation that the AmEPV derived colonies gradually became more resistant to G418 and that GFP expression while delayed, is ultimately observed. Fluorescent cells became both more numerous and brighter, consistent with a gradual increase in GFP concentration with time. Ultimately all cells in each clonal isolate were GFP positive, as shown in Figure 6.

We are currently characterizing these cell lines, which have been grown reliably for multiple generations. Our unpublished data suggests random integration of the marker cassette into the cellular genome. Inclusion of the AAV *rep* gene in future constructs is anticipated to provide directed insertion into chromosome 19 (Samulski, R.J., X. Zhu, X. Xiao, J.D. Brook, D.E. Housman, N. Epstein and L.A. Hunter [1991] "Targeted integration of adeno-associated virus (AAV) into human chromosome 19" *EMBO J.* 10:3941-3950).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

We claim:

1. A method for providing a vertebrate animal with a therapeutically effective amount of a protein, said method comprising introducing into cells of said animal an effective amount of a recombinant entomopox virus vector, wherein said vector comprises a polynucleotide encoding said protein.

2. The method according to claim 1, wherein said animal is a mammal.

3. The method according to claim 2, wherein said mammal is a human.

4. The method according to claim 1, wherein said vector comprises inverted terminal repeat sequences flanking said polynucleotide encoding said protein.

5. The method according to claim 4, wherein said inverted terminal repeat sequences are derived from adeno-associated virus.

6. The method according to claim 1, wherein said vector comprises a promoter sequence capable of driving expression of said polynucleotide encoding said protein.

7. The method according to claim 6, wherein said promoter sequence is selected from the group consisting of a CMV promoter sequence and herpes TK promoter sequence.

8. The method according claim 1, wherein said protein encoded by said polynucleotide is selected from the group consisting of interleukins, cytokines, growth factors, interferons, enzymes and structural proteins.

9. The method according to claim 1, wherein said vector is introduced into said cells of said animal by infection in a viral particle.

10. The method according to claim 1, wherein said vector is introduced into said cells of said animal by means selected from the group consisting of transfection, transduction and injection.

11. The method according to claim 1, wherein said vector is introduced into said cells of said animal *in vitro* and said treated cells are introduced into said animal.

12. The method according to claim 1, wherein said vector is introduced into said cells of said animal *in vivo*.

13. The method according to claim 1, wherein said polynucleotide encoding said protein is greater than about 10 kb in size.

14. The method according to claim 1, wherein said polynucleotide also encodes a selectable marker protein.

15. A recombinant entomopox virus vector comprising a polynucleotide encoding a protein capable of providing a therapeutic effect to an animal when expressed in said animal.

16. The recombinant vector according to claim 15, wherein said animal is a mammal.

17. The vector according to claim 16, wherein said mammal is a human.

18. The vector according to claim 15, wherein said entomopox virus is *Amsacta moorei*.

19. The vector according to claim 15, wherein said vector comprises inverted terminal repeat sequences flanking said polynucleotide encoding said protein.

20. The vector according to claim 19, wherein said inverted terminal repeat sequences are derived from adeno-associated virus.

21. The vector according to claim 15, wherein said vector comprises a promoter sequence capable of driving expression of said polynucleotide encoding said protein.

22. The vector according to claim 21, wherein said promoter sequences are selected from the group consisting of CMV and herpes TK.

23. The vector according to claim 15, wherein said protein encoded by said polynucleotide is selected from the group consisting of interleukins, cytokines, growth factors, interferons, enzymes and structural proteins.

24. The vector according to claim 15, wherein said polynucleotide encoding said protein is greater than about 10 kb in size.

25. The vector according to claim 15, wherein said polynucleotide also encodes a selectable marker protein.

26. A composition of matter comprising a recombinant entomopox vector, wherein said vector comprises a polynucleotide encoding a protein capable of providing a therapeutic effect to an animal when expressed in said animal, and

wherein said composition of matter is selected from the group consisting of viral particles and cells.

27. The cell according to claim 26, wherein said cell expresses a protein encoded by said polynucleotide.

28. An isolated polynucleotide encoding an *Amsacta moorei* entomopox virus protein selected from the group consisting of triacylglyceride lipase, Cu^{++}/Zn^{++} superoxide dismutase, CPD photolyase, baculovirus-like inhibitor of apoptosis, first poly(A) polymerase small subunit, second poly(A) polymerase small subunit, first DNA polymerase, second DNA polymerase, ABC transporter-like protein, Kunitz-motif protease inhibitor and poly(A) polymerase large subunit.

29. The polynucleotide according to claim 28, wherein said triacylglyceride lipase comprises SEQ ID NO: 12 or a fragment or variant thereof.

30. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 1 or a fragment or variant thereof.

31. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 1 or its complement.

32. The polynucleotide according to claim 28, wherein said Cu^{++}/Zn^{++} superoxide dismutase comprises SEQ ID NO: 13 or a fragment or variant thereof.

33. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 2 or a fragment or variant thereof.

34. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 2 or its complement.

35. The polynucleotide according to claim 28, wherein said CPD photolyase comprises SEQ ID NO: 14 or a fragment or variant thereof.

36. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 3 or a fragment or variant thereof.

37. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 3 or its complement.

38. The polynucleotide according to claim 28, wherein said baculovirus-like inhibitor of apoptosis comprises SEQ ID NO: 15 or a fragment or variant thereof.

39. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 4 or a fragment or variant thereof.

40. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 4 or its complement.

41. The polynucleotide according to claim 28, wherein said first poly(A) polymerase small subunit comprises SEQ ID NO: 16 or a fragment or variant thereof.

42. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 5 or a fragment or variant thereof.

43. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 5 or its complement.

44. The polynucleotide according to claim 28, wherein said second poly(A) polymerase small subunit comprises SEQ ID NO: 17 or a fragment or variant thereof.

45. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 6 or a fragment or variant thereof.

46. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 6 or its complement.

47. The polynucleotide according to claim 28, wherein said first DNA polymerase comprises SEQ ID NO: 18 or a fragment or variant thereof.

48. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 7 or a fragment or variant thereof.

49. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 7 or its complement.

50. The polynucleotide according to claim 28, wherein said second DNA polymerase comprises SEQ ID NO: 19 or a fragment or variant thereof.

51. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 8 or a fragment or variant thereof.

52. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 8 or its complement.

53. The polynucleotide according to claim 28, wherein said ABC transporter-like protein comprises SEQ ID NO: 20 or a fragment or variant thereof.

54. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 9 or a fragment or variant thereof.

55. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 9 or its complement.

56. The polynucleotide according to claim 28, wherein said Kunitz-motif protease inhibitor comprises SEQ ID NO: 21 or a fragment or variant thereof.

57. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 10 or a fragment or variant thereof.

58. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 10 or its complement.

59. The polynucleotide according to claim 28, wherein said poly(A) polymerase large subunit comprises SEQ ID NO: 22 or a fragment or variant thereof.

60. The polynucleotide according to claim 28, wherein said polynucleotide comprises SEQ ID NO: 11 or a fragment or variant thereof.

61. The polynucleotide according to claim 28, wherein said polynucleotide hybridizes with the sequence as set forth in SEQ ID NO: 11 or its complement.

62. An isolated *Amsacta moorei* entomopox virus protein selected from the group consisting of triacylglyceride lypase, Cu^{++}/Zn^{++} superoxide dismutase, CPD

photolyase, baculovirus-like inhibitor of apoptosis, first poly(A) polymerase small subunit, second poly(A) polymerase small subunit, first DNA polymerase, second DNA polymerase, ABC transporter-like protein, Kunitz-motif protease inhibitor and poly(A) polymerase large subunit.

63. The triacylglyceride lipase of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 12, or a fragment or variant thereof.

64. The Cu^{++}/Zn^{++} superoxide disumutase of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 13, or a fragment or variant thereof.

65. The CPD photolyase of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 14, or a fragment or variant thereof.

66. The baculovirus-like inhibitor of apoptosis of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 15, or a fragment or variant thereof.

67. The first poly(A) polymerase small subunit of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 16, or a fragment or variant thereof.

68. The second poly(A) polymerase small subunit of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 17, or a fragment or variant thereof.

69. The first DNA polymerase of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 18, or a fragment or variant thereof.

70. The second DNA polymerase of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 19, or a fragment or variant thereof.

71. The ABC transporter-like protein of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 20, or a fragment or variant thereof.

72. The Kunitz-motif protease inhibitor of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 21, or a fragment or variant thereof.

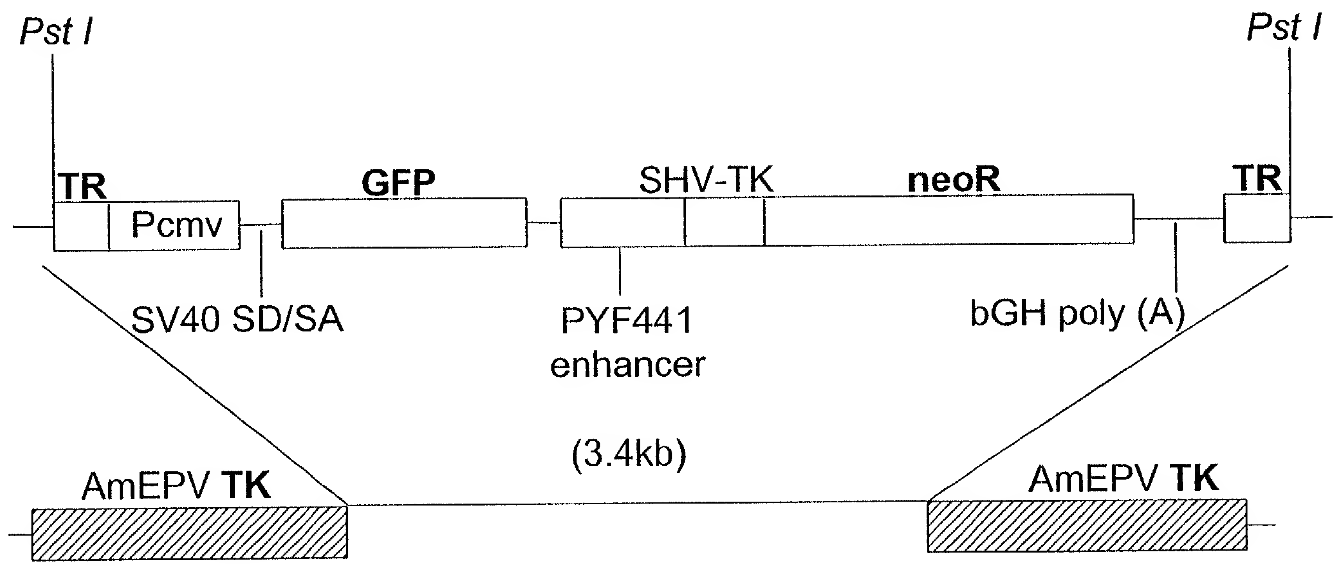
73. The poly(A) polymerase large subunit of claim 62 comprising the amino acid sequence as set forth in SEQ ID NO: 22, or a fragment or variant thereof.

74. An isolated polynucleotide encoding an *Amsacta moorei* entomopox virus polypeptide, wherein said polynucleotide is selected from the group consisting of AMVITR10, AMV002, AMV047, AMV051, AMV054, AMV059, AMV061, AMV066, AMV078, AMV079, AMV081, AMV084, AMV087, AMV91, AMV093, AMV105, AMV114, AMV122, AMV135, AMV139, AMV147, AMV150, AMV153, AMV166, AMV167, AMV174, AMV181, AMV192, AMV193, AMV197, AMV199, AMV205, AMV221, AMV228, AMV230, AMV231, AMV234, AMV246, AMV248 and AMV256, or a fragment or variant thereof.

75. An isolated *Amsacta moorei* entomopox virus polypeptide encoded by a polynucleotide selected from the group consisting of AMVITR10, AMV002, AMV047, AMV051, AMV054, AMV059, AMV061, AMV066, AMV078, AMV079, AMV081, AMV084, AMV087, AMV91, AMV093, AMV105, AMV114, AMV122, AMV135, AMV139, AMV147, AMV150, AMV153, AMV166, AMV167, AMV174, AMV181, AMV192, AMV193, AMV197, AMV199, AMV205, AMV221, AMV228, AMV230, AMV231, AMV234, AMV246, AMV248 and AMV256, or a fragment or variant thereof.

Abstract of the Disclosure

The subject invention pertains to recombinant entomopox vectors which are useful for the delivery and stable expression of heterologous DNA in vertebrate cells. Specifically exemplified is a recombinant EPV from *amsacta moorei* (AmEPV). Because of the capacity of the EPV to incorporate foreign or heterologous DNA sequences, the vectors of the subject invention can be used to deliver DNA inserts that are larger than 10 kb in size. Accordingly, one aspect of the present invention concerns use of the recombinant vectors for delivery and expression of biological useful proteins in gene therapy protocols. In addition, the subject invention concerns novel AmEVP polypeptides and the polynucleotide sequences which encode these polypeptides.


Pst I
Pst I
TR
Pcmv
GFP
SHV-TK
neoR
TR
SV40 SD/SA
PYF441
enhancer
bGH poly (A)
(3.4kb)
AmEPV **TK**
AmEPV **TK**

pTKUF5

FIG. 1

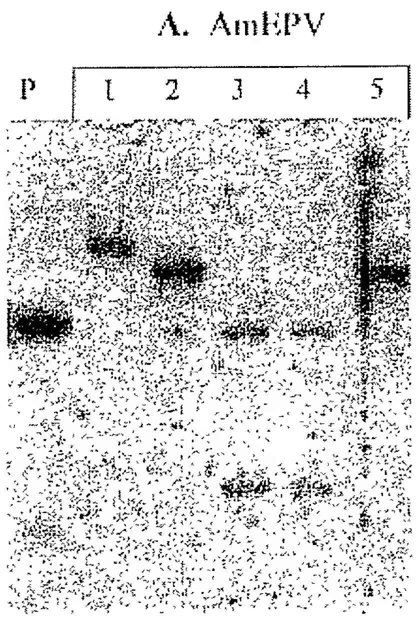


FIG. 2A

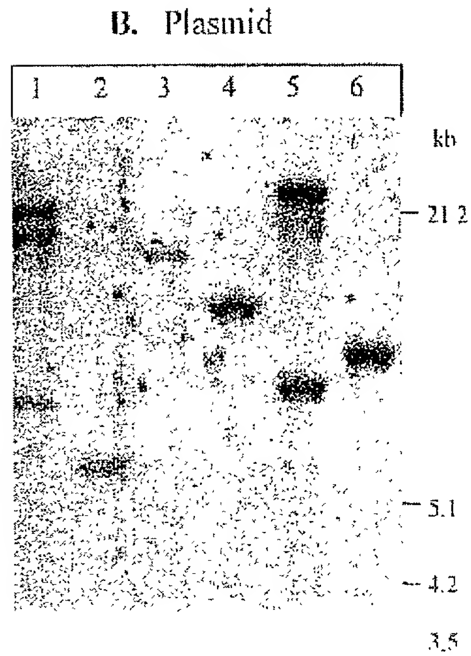


FIG. 2B

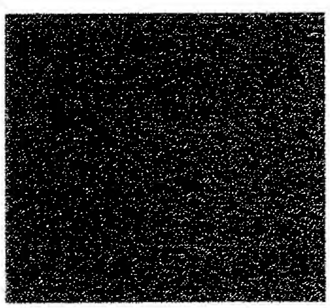


FIG. 3A

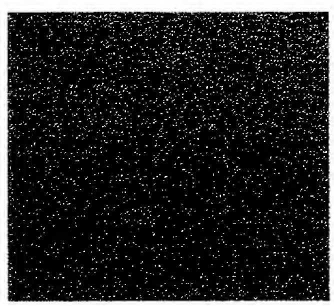


FIG. 3B

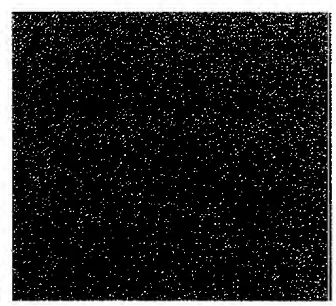


FIG. 3C

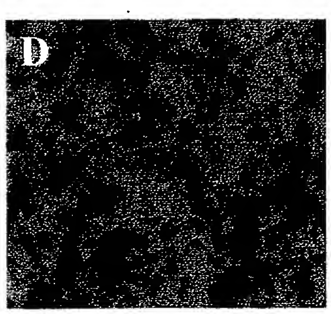


FIG. 3D

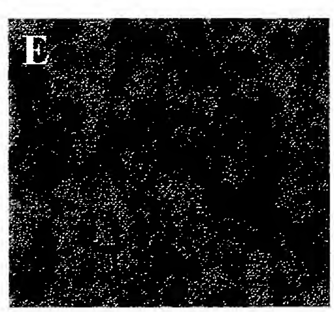


FIG. 3E

FIG. 3F

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

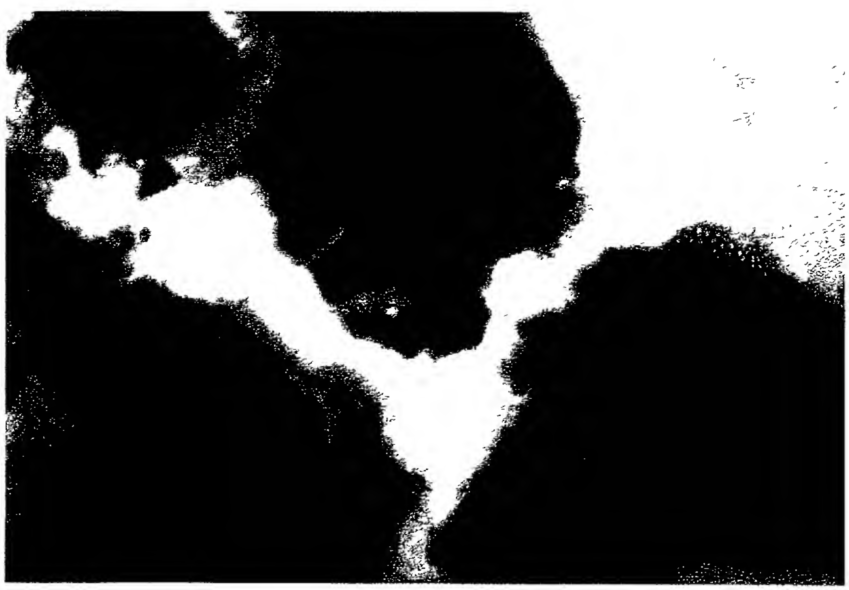


FIG. 5

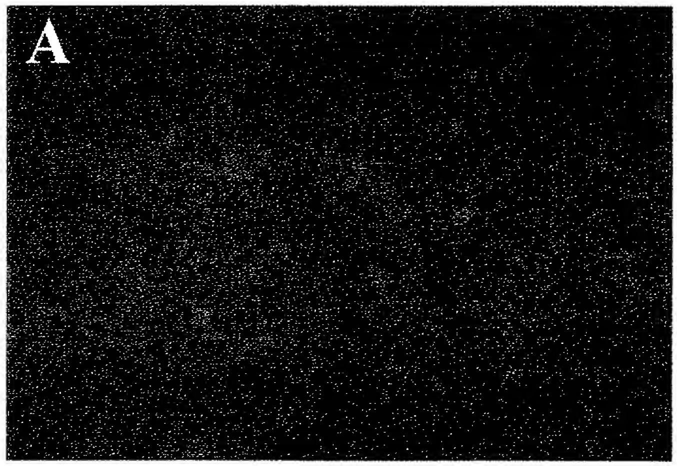


FIG. 6A

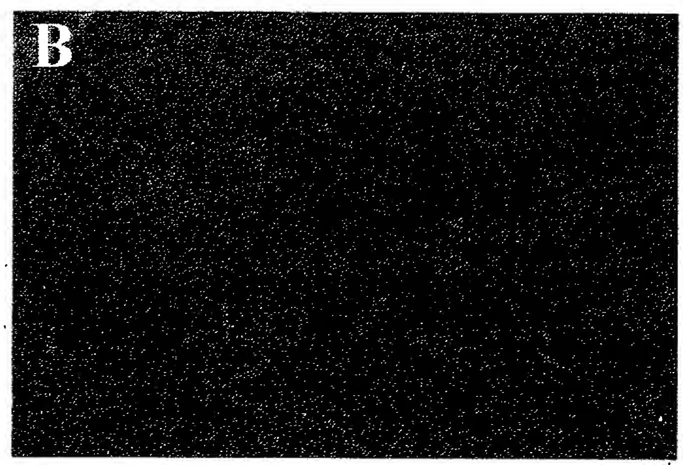


FIG. 6B

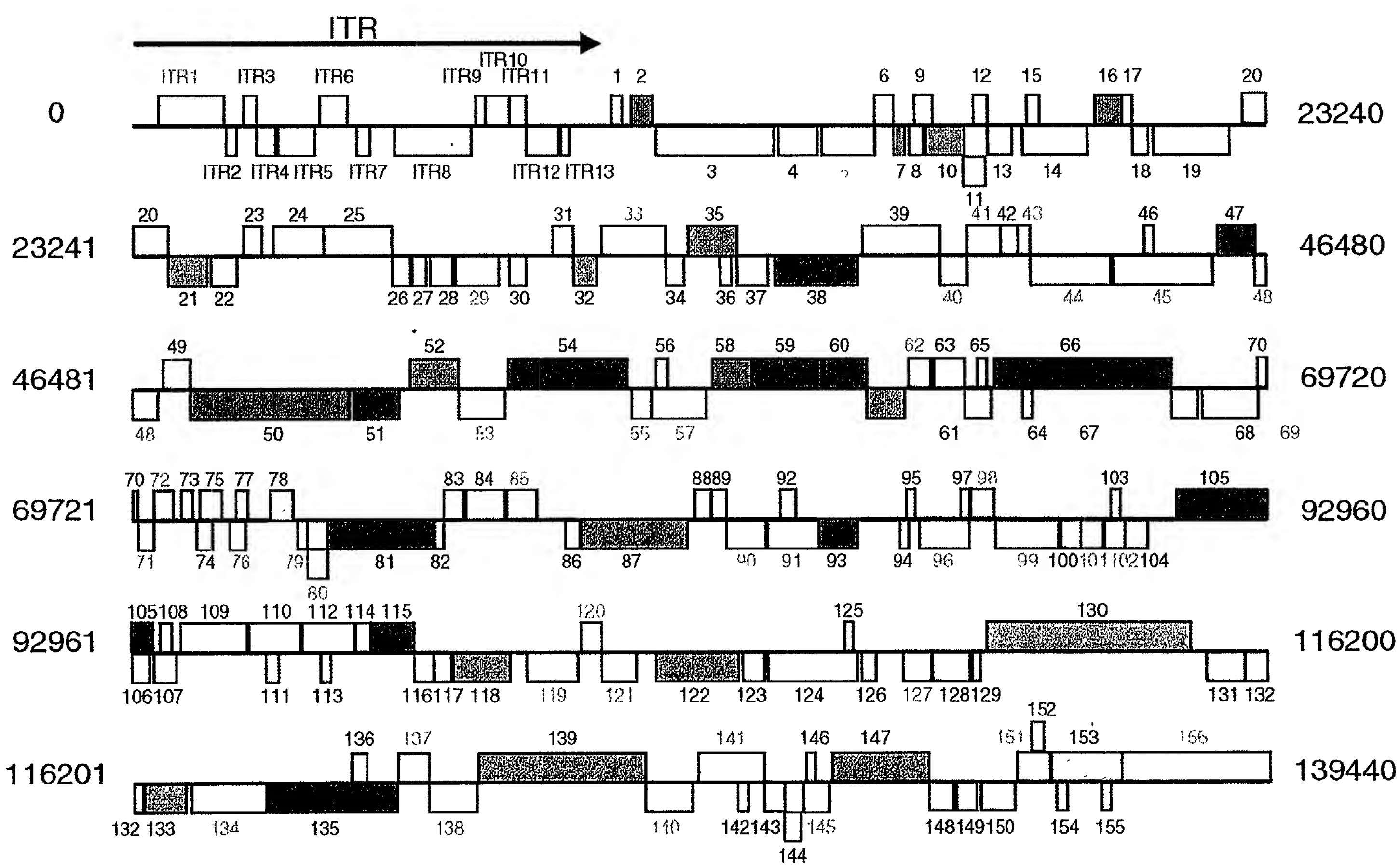
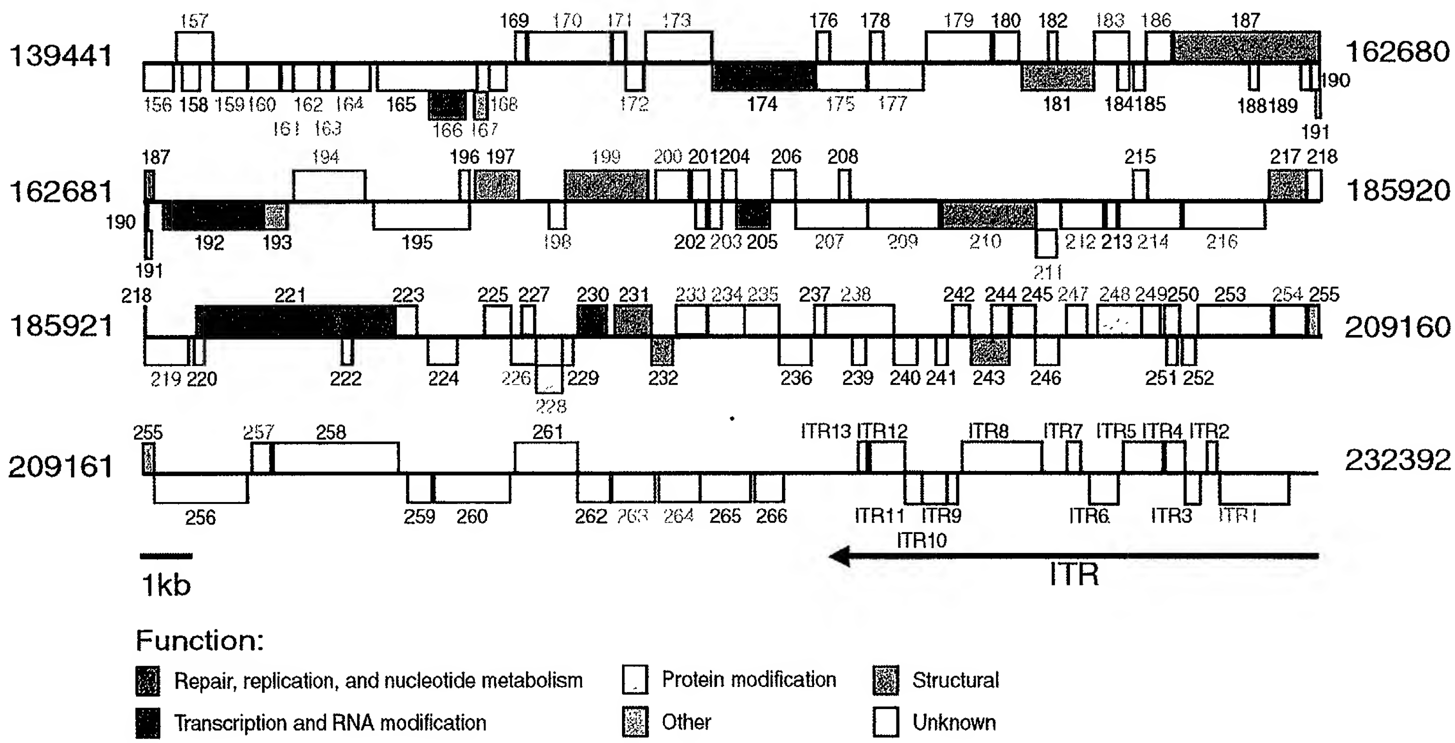

ITR
0
23240
23241
46480
46481
69720
69721
92960
92961
116200
116201
139440

FIG. 7A



139441
162680
162681
185920
185921
209160
209161
232392
ITR
1kb
Function:
Repair, replication, and nucleotide metabolism
Transcription and RNA modification
Protein modification
Other
Structural
Unknown

FIG. 7B

FIG. 8A

FIG. 8B

FIG. 8C

FIG. 8D

FIG. 9A

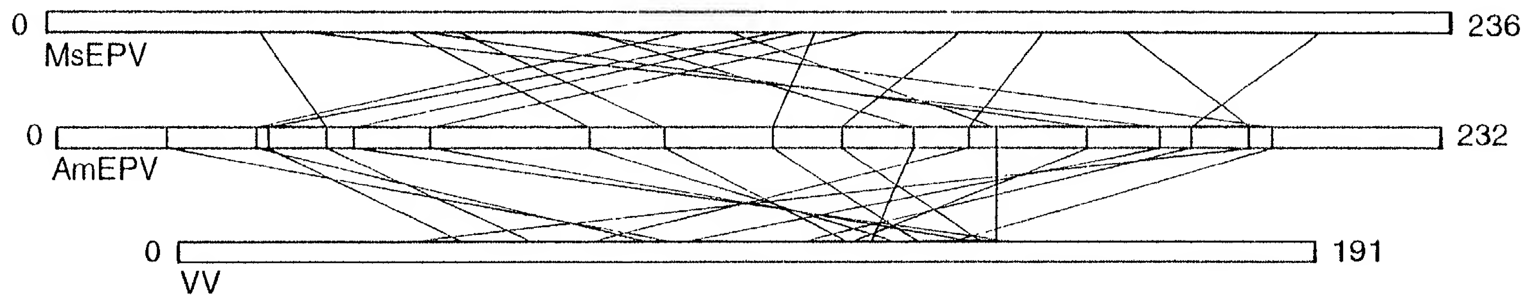
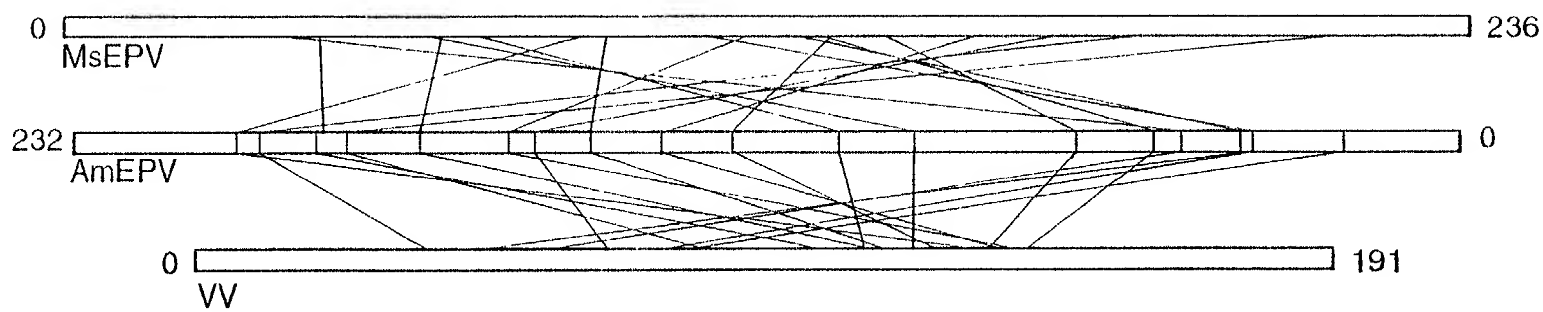


FIG. 9B



```
           1                                                  50                                                                                                 100
     HaEPV        MSF .NPIIYYISD I...KNERPY KKNTKP.YIF NFRKPGQIKL LINEIRFLTE .DVEIYKNYN NKIINILYIG SGKGYHIPLL MEIYSKY.N.
    AMV060        MNF .MPQYYYISD I...NNEIEY DENFNPGKKF DFKRQGQIKL LMNEIRFLTE .DVELHKNYK NENINILYIG SGKGYHIPLL INMYSDY.K.
    MSV041 MSIYMKINDF KKPNVLIFDN I...NNQLKY ....KPNNVS N.KHPGQLKL LMTELQFFNN .CNIDALNSK DRPIYVLYIG SGRGYHLIKL LDLYKDY.N.
    AMV115 MDVNKYIYEY NKPLYYTYYD LCRNMNDVIY DYNNNTIKKY .......MDI LLSQIQFLSN INIKKICNNT NGIVNTLYIG SSKAYHFNIL NELYKNLTN.
     VVJ3R     MDVVSL DKP.FMYFEE I...DNELDY EPESANEVAK KLPYQGQLKL LLGELFFLSK LQRHGILDG. ...ATVVYIG SAPGTHIRYL RDHFYNLGMI
 Consensus        --- --P------- -----N---Y ---------- ---------- L-----F--- ---------- -------YIG S----H---L ----------
Insect Cons       --- --P------- -----N---Y ---------- ---------- L-----F--- -------N-- ------LYIG S---YH---L ---Y------
AmEPV Cons        --- --P-YY---D -----N---Y D-N-N--KK- ---------- L---I-FL-- -------N-- N---NILYIG S-K-YH---L ---Y------

           101                                                150                                                                                                200
     HaEPV IIWHLYDPNG HCDKLNEISN KNNNVNIYNQ IFDKKDVELY EDVQNLLFIS DIRTIDDDKI EPNTKNLIHD YDIQNYVLKQ LKPI.ALIKQ RDPFPNDWDE
    AMV060 IQWDLYDPCG HCEKLYNIQK NNNNIKIYDT YFNKSDVEKY ENIDNLLFIT DIRTVDHPDD EPNTKNLIND YELQNYILKE LKPI.SLVKQ RDPFPNDWDD
    MSV041 IKWYFYDPSG HCISLERMSQ ...YVSINND YFTEKNINEF KNKKPLLFIS DIRSTDGS.. EPRTKNLIDD YKIQNNIVLN LRPLYSLLKF RYPFPDDFPP
    AMV115 IQWYFYDIID PCISVERLS. ..YNIIFNRK LFTEDDIIDF KDKYPLILIY DY......DD KSNVRDLLYH YNMQNNIIIY LNPTYSLLKF KYMPINKWNN
     VVJ3R IKWMLIDGRH HDPILNGLRD VTLVTRFVDE EYLRS.IKKQ LHPSKIILIS DVRSKRGGN. EPSTADLLSN YALQNVMISI LNPVASSLKW RCPFPDQWIK
 Consensus I-W---D--- ---------- ---------- ---------- ---------I- D--------- ------L--- Y--QN----- L-P-----K- ----------
Insect Cons I-W--YD--- -C-------- ---------- -F-------- -----L--I- D--------- ------L--- Y--QN----- L-P---L-K- ----------
AmEPV Cons IQW--YD--- -C-------- ---NI----- -F---D---- -----L--I- D-------DD --N---L--- Y--QN-I--- L-P--SL-K- -----N-W--

           201                                                250                                                                                                300
     HaEPV SYEMYI.PD. GKEYVQCFQK HDSAEYRIF. VCGATTFTKV NLDVLKTRNI DKKLAWYNTK YRFDNFNDY. .RIAYRVLNK YLKTENLPFL .KYTDINKNN
    AMV060 SYKLSI.PD. GKEYIQCFQK YNSAEYRIF. ISGITTFVDI NSVILNKRGI DRKLAWYNMK YRFQNDNDY. .KIAYRILNK YIKSENKPIL KKYNNTNKNN
    MSV041 EIENEVYVD. GTKFLQPFCG PQSTEMRIF. VSEQNIILK. NFSKEESILF EEKMYYYNKN YRIINKNDI. .LIAGFILKS TNKFDNM... .KYIDIIKSL
    AMV115 SFNDYEYIST GIKYLPTI.. .KSLHTRNI. IDNKNIMT.L TFDEIESENY YEKMNYYN.. .NCSGYNDIY NNISGYILNK SNLYDNNN.. SAY.NILKIY
     VVJ3R DF....YIPH GNKMLQPFAP SYSAEMRLLS IYTGENMRLT RVTKSDVVNY EKKMYYLNKI VR....NKV. ......VVNF DYPNQEYDYF HMYFMLRTVY
 Consensus ---------- G--------- --S---R--- ---------- ---------- --K----N-- ------N--- ---------- ---------- --Y-------
Insect Cons ---------- G--------- --S---R--- ---------- ---------- --K---YN-- ------ND-- --I----L-- -----N---- --Y--I-K--
AmEPV Cons S--------- G--Y------ --S---R--- I--------- ---------- --K---YN-- ------ND-- --I---ILNK -----N---- --Y-NI-K--

           301                                                350                  362
     HaEPV IKSVIKSISK TINDN
    AMV060 IKNVIRSLSK EMGYY
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    AMV115 EKNIINTINE DKIFRSKEKY I
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FIG. 10

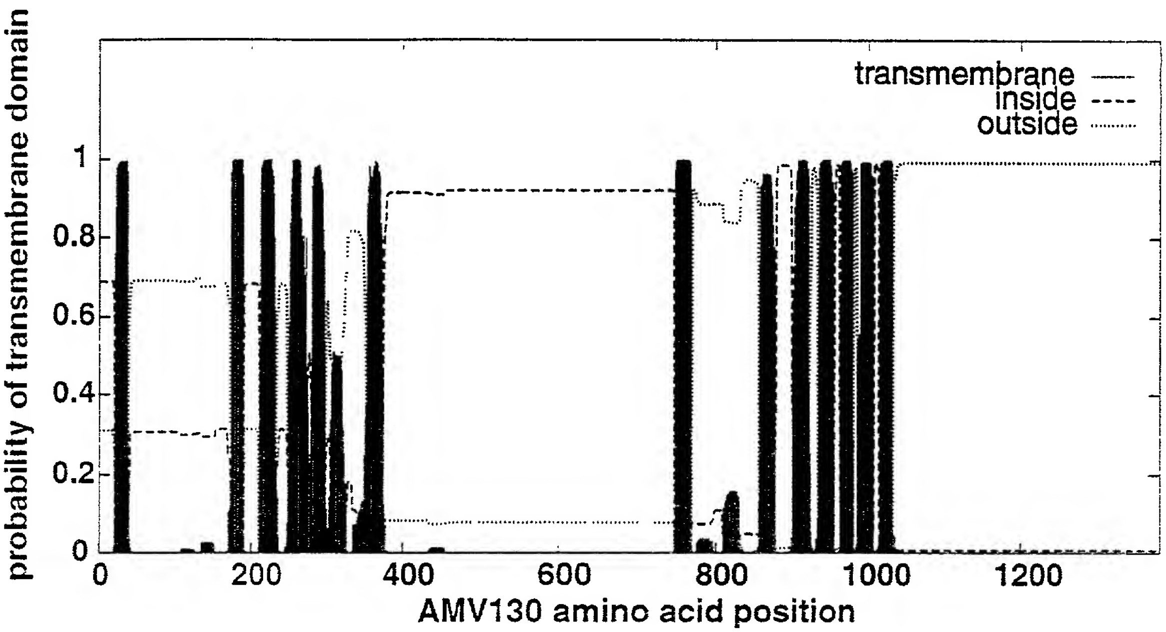

FIG. 11

1 MNYYILLCLF MLFSSSYNFK LINNNICNED YDPGICRIGD
41 IRWYYNYNIK DCKI*FIYGGC GGNMNNFNNY EDC*INKCLI

FIG. 12

Docket No. UF-221C1XC1

DECLARATION (37 CFR 1.63) AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name; and

I believe that I am the original, first, and sole inventor (if only one name is listed below), or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **Materials And Methods For Delivery And Expression Of heterologous DNA In Vertebrate Cells**, specification for which

☒ is attached hereto.

☐ was filed ____________________, Serial No. ____________.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code §119 and/or §365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Application Serial No.	Country	Filing Date	Priority Claimed

I hereby claim priority benefits under Title 35, United States Code §119 of any provisional application(s) for patent listed below:

Application Serial No.	Filing Date	Priority Claimed
60/224.479	10 August 2000	Yes

I hereby claim the benefit under Title 35, United States Code, §120 and/or §365 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (Patented, Pending, Abandoned)
09/086,651	29 May 1998	pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following persons registered to practice before the Patent and Trademark Office as my attorneys with full power of substitution and revocation to prosecute this application and all divisions and continuations thereof and to transact all business in the Patent and Trademark Office connected therewith: David R. Saliwanchik, Reg. No. 31,794; Jeff Lloyd, Reg. No. 35,589; Doran R. Pace, Reg. No. 38.261; Christine Q. McLeod, Reg. No. 36.213; Jay M. Sanders, Reg. No. 39,355; James S. Parker, Reg. No. 40,119; Frank C. Eisenschenk, Reg. No. 45,332; Jean Kyle, Reg. No. 36,987; Seth M. Blum, Reg. No. 45,489; Glenn P. Ladwig, Reg. No. P-46,853.

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Date ____________

Signature of First or Sole Inventor

****

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Chomedey, Lazal, H7 V1J7 Canada

Date ____________

Signature of Second Joint Inventor

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Gainesville, FL 32607

Date ____________

Signature of Third Joint Inventor

****

Name of Fourth Joint Inventor ____________

Residence ____________ Citizenship ____________

Post Office Address ____________

Date ____________

Signature of Fourth Joint Inventor

SEQUENCE LISTING

<110> Moyer, Richard W.
Yi, Li
Bawden, Alison L.

<120> Materials and Methods for Delivery and Expression of Heterologous DNA in Vertebrate Cells

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tttgaccaat taaattatgt agaaaaacaa aataaaagtt taacatttaa taatattaat 1020

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tatatgaaaa tgaaatatat atatattata aatgaaaata ataattatat gttagaaaca 1200

gaaaataatg attattatgt taaaatacaa aacatatata aatattatga taataatttt 1260

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ggtaaaatat taactaataa tatcaaaata ggttattgtc cacaaaataa tataaatttt 1440

acagatttta ctgtaaaaga aaatatatat ttatttaata tattgagagg attaagttcg 1500

ttacaatcaa aaataaaaac aaatgaaata attatttatc taaaattaca tgatatagaa 1560

aattgtataa taacagaatt atctgaatgt tcaaaacgta aattacaatt agctttttcg 1620

ttaatagatg attctgattt tatattaatc gatgaaccca cacataatat agatttaaaa 1680

agtaaacaag aaatatggga tttaatatca ttattaaaaa gaaataaaac tatattaatt 1740

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ggaaatgtta aatataattc gacattattt aatattaaaa aagatgcaaa tgtaacttat 1860

aaattatcaa tacataataa ttctaccgac gataaaataa aaaatataat tattaatagt 1920

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caattatttt ca 4152

<210> 10

<211> 236

<212> DNA

<213> Amsacta moorei entomopoxvirus

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<210> 11

<211> 1719

<212> DNA

<213> Amsacta moorei entomopoxvirus

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ataaaaagaa gaataacaga attttttgat aaacaaaata cgccaaattt aaaattagga 240

agtataatat caattattaa atttcaacat ttaactgtaa catatgttaa taaaataata 300

aaagaaattg taacatataa atgtaatact agagaaataa atatagtaaa tttttcatct 360

gtcacatctc aaatttcaaa ctacgataat cctatattaa atgaaatatt aaaacaatat 420

gtatataaac aaaaattaaa aaatgttact gttaataatg ataaaaagaa aataattgat 480

cctgatgatg agaaattagc tgaatctatt aaaaaaatat tagaagaaat attaaaaata 540

ttattaatta taaaaaacaa tgattgtgtt gcttatgggt catttacttg ttataatata 600

aatagaagta taaaatataa tgatatagat ttatatagta ctgatgcata tagaatttta 660

atatttttta tgatatatat acatttaact attggacatg acacttgttt atttagtata 720

ccttttataa ctgggcacat atcgttaaaa tataaaaata tatttataat agattgtata 780

tttttagata attctattat aaatgttatt aataaatctt taattaataa tatatatttt 840

atagatcccg gtttacaaat gttaaataat tttagaatgt tatcagaaaa ttttagatct 900

tataaaatat atgaaaaaat ggaagaatct ttaaataaat ataaaacatt attaaattat 960

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gaattgatag atataagtcc atatgattat ataatgattg tattagattc gccgtcagac 1140

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aatgaaatat tttttgaaac aaaaaaaata aaaaataaaa taaatacata tgctggaaac 1260

acaaataaca taacacaatt aattgatgaa aataaattaa taaaattaaa tagaagtgat 1320

ataaatatgc catataatat taatcccaat aagaaatatt taattttcag taatttaaca 1380

acatctacgt atgtttactt tgagaatgat aaaatgactg atatatcagt aaaaaatcta 1440

atatcattta tatcaacagc ttgtttatat aatttgttac acaaaaaaga tgattttggt 1500

atggaattat attatttaac attacactgt cttacattta ccgaaactag aaaattaaat 1560

gaatataaag taatagatag atataaaata aaaggcgaac ataaagaaat atcattgtgt 1620

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aatacattta tagatttaac taatataaat ggaggatat 1719

<210> 12

<211> 286

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 12

Met Thr Ile Phe Glu Ile Leu Ile Trp Ile Ile Val Leu Leu Ala Phe
1 5 10 15

Met Phe Ile Ile Phe Leu Tyr Val Val Leu Tyr Ile Lys Arg Arg Ile
20 25 30

Tyr Glu Ile Leu Asn Glu Asn Ile Pro Ile Glu Ile Asn Ile Asp Asn
35 40 45

Val Asn Tyr Pro Ser Glu Leu Tyr Thr Asp Lys Phe Asn Pro Asn Val
50 55 60

Leu Lys Tyr Leu Ile Lys Ile Leu Leu Asp Phe Asn Thr Glu Ile Thr
65 70 75 80

Asn Asn Ile Ile Ile His Ser Ile Asp Tyr Met Lys Ile Tyr Tyr Ile
85 90 95

Ser Tyr Asn Lys Lys Lys Ile Ile Lys Leu Ile Leu Asp Arg Tyr Asn
100 105 110

Asn Leu Trp Ile Val Ile Arg Gly Thr Leu Thr Tyr Asn Glu Phe Glu
115 120 125

His Asp Leu Arg Ile Ser Gln Val Lys Ile Asp Asn Cys Asp Met Lys
130 135 140

Cys His Lys Gly Phe Cys Glu Ile Tyr Ser Lys Ile Gln Lys Pro Leu
145 150 155 160

Leu Asn Leu Leu Met Thr Leu Ser Pro Asn Lys Ile Phe Ala Leu Gly
165 170 175

His Ser Leu Gly Gly Gly Ile Leu Ser Ile Ala Ala Tyr Asp Ile Phe
180 185 190

Asn Ile Leu Asn Lys Lys Glu Ile Ile Leu Tyr Thr Thr Gly Thr Pro
195 200 205

Arg Val Cys Asn Lys Asp Phe Tyr Asn Asn Cys Asn Lys Tyr Asn Ile
210 215 220

His Lys Val Glu Asn Leu Ser Asp Val Tyr Ile Asn Ala Ile Pro Ser
225 230 235 240

Val Leu Pro Phe Tyr Asp Asn Thr Val Tyr Tyr Lys Ile Gly Lys Ile
245 250 255

Trp Tyr Phe Asp Val Asn Tyr Gly Asn Ile Ile Leu His Lys Leu Glu
260 265 270

Ile Tyr Phe Asn Asn Ile Asp Asn Leu Lys Tyr Leu Glu Ile
275 280 285

<210> 13

<211> 151

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 13

Met Lys Ala Ile Cys Val Met Thr Gly Lys Val Asn Gly Ile Ile Tyr
1 5 10 15

Phe Ile Gln Asn Ile Lys Gly Gly Ser Val His Val Lys Gly Lys Ile
20 25 30

Val Gly Leu Ser Lys Gly Leu His Gly Phe His Val His Glu Tyr Gly
35 40 45

Asp Val Ser Asn Gly Cys Thr Ser Ala Gly Glu His Phe Asn Pro Tyr
50 55 60

Asn Arg Gln His Gly Asp Ile Ser Asp Lys Ile His Arg His Val Gly
65 70 75 80

Asp Phe Gly Asn Val Tyr Ala Asp Glu Asn Gly Val Ala Asn Ile Asp
85 90 95

Phe His Asp Asp Ile Ile Ser Leu Cys Gly Thr Asn Asn Ile Ile Gly
100 105 110

Arg Thr Leu Val Val His Asp Ser Pro Asp Asp Leu Gly Lys Thr Asp
115 120 125

Pro Leu Ser Lys Thr Ser Gly Asn Ser Gly Gly Arg Leu Gly Cys Gly
130 135 140

Ile Ile Gly Ile Ala Lys Asp
145 150

<210> 14

<211> 453

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 14

Met Tyr Asn Asn Glu Tyr Phe Thr Asn Arg Val Lys Ile His Lys Lys
1 5 10 15

Ile Asp Thr Ile Asn Lys Asn Val Leu Tyr Leu Ala Tyr Arg Asp Leu
20 25 30

Arg Val Tyr Asp Asn Trp Ser Phe Leu Tyr Ser Gln Asn Ile Ala Tyr
35 40 45

Leu Asn Asn Ser Ser Met Tyr Val Leu Tyr Leu Ile Asn Lys Asn Asn
50 55 60

Asn Ile Asn Ile Arg Gln Tyr Lys Phe Leu Tyr Glu Gly Leu Pro Glu
65 70 75 80

Phe Glu Ser Gln Cys Lys Lys Cys Asn Val Ser Phe His Leu Leu Ser
85 90 95

Tyr Asn Asn Asn Ile Ile Ser Asn Phe Ile Asn Lys Tyr Lys Ile Gly
100 105 110

His Val Ile Ile Glu Gln Met Pro Leu Leu Phe His Lys Lys Tyr Tyr
115 120 125

Leu Asp Pro Leu Lys Lys Leu Asn Val Asn Val Tyr Ile Val Asp Ser
130 135 140

His Asn Ile Ile Pro Val Trp Val Thr Ser Asp Lys Gln Glu Tyr Asn
145 150 155 160

Ala Arg Thr Ile Arg Ile Lys Ile Asn Lys Leu Lys Asp Gln Tyr Leu
165 170 175

Ile Glu Phe Pro Lys Val Lys Ile Ser Asn Ile Gln Pro Ile Phe Val
180 185 190

Glu Asn Asn Phe Asp Ile Ile Pro Asn Tyr Asp Lys Lys Leu Ile Asn
195 200 205

Ile Tyr Glu Ile Val Gly Gly Tyr Thr Asn Gly Ile Asn Arg Met Asn
210 215 220

Asn Phe Phe Lys Asn Lys Ile Asn Thr Tyr Lys Asp Lys Lys Asn Asn
225 230 235 240

Pro Asn Tyr Glu Asn Thr Ser Ile Leu Ser Pro Trp Leu His Cys Gly
245 250 255

Met Ile Ser Ala Gln Arg Cys Val Leu Glu Ala Asn Lys Leu Lys Lys
260 265 270

Ile Lys Asp Tyr Asn Ile Glu Ser Ile Asp Ser Phe Ile Glu Glu Ile
275 280 285

Phe Ile Arg Lys Glu Leu Ser Asp Asn Phe Cys Tyr Tyr Asn Asn Asn
290 295 300

Tyr Lys Ser Phe Ala Ser Cys Pro Asn Trp Ala Ile Leu Thr Leu Glu
305 310 315 320

Ile His Lys Thr Asp Lys Arg Asn Lys Ile Phe Ser Leu Arg Glu Leu
325 330 335

Glu Tyr Gly Lys Thr Asp Asn Lys Leu Trp Asn Tyr Cys Gln Tyr Tyr
340 345 350

Leu Leu Lys Phe Gly Tyr Leu Asn Gly Tyr Met Arg Met Phe Trp Ala
355 360 365

Lys Lys Leu Ile Glu Trp Thr Asn Ser Pro Gln Asp Ala Ile Asp Lys
370 375 380

Thr Ile Tyr Leu Asn Asp Lys Tyr Phe Phe Asp Gly Tyr Asp Pro Met
385 390 395 400

Gly Tyr Val Asn Ile Leu Trp Ser Ile Gly Gly Leu His Asp Arg Ala
405 410 415

Phe Lys Glu Arg Glu Met Tyr Gly Lys Ile Arg Phe Met Ser Gln Pro
420 425 430

Leu Met Tyr Lys Lys Leu Asn Val Asn Asp Phe Tyr Asn Asn Phe Asp
435 440 445

Asn Val Ile Lys Ser
450

<210> 15

<211> 263

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 15

Met Met Asp Asp Ile Asn Leu Tyr Asn Glu Ser Glu Arg Leu Gln Thr
1 5 10 15

Phe Glu Asn Trp Pro Ile Asn Phe Ile Thr Pro Glu Ser Phe Ala Ser
20 25 30

Asn Gly Phe Tyr Tyr Ile Gly Glu Asn Asp Thr Val Lys Cys Val Tyr
35 40 45

Cys Gly Val Gln Ile Asn Lys Trp Val Glu Gly Asp Lys Pro Glu Ile
50 55 60

Asp His Lys Lys Phe Ser Pro Asn Cys Ser Phe Leu Lys Ser Asn Asp
65 70 75 80

Gly Ile Asp Glu Cys Gly Asn Asn Lys Asn Ile Ser Asn Ile Thr Gln
85 90 95

Lys Gly Ala Val His Pro Asn Leu Ser Asn Ile Val Glu Arg Leu Lys
100 105 110

Thr Tyr Lys Glu Trp Pro Ile Ser Met Pro Ile Ser Thr Glu Lys Leu
115 120 125

Glu Ala Gly Phe Phe Tyr Thr Gly Lys Ser Asp Lys Val Lys Cys Phe
130 135 140

Tyr Cys Asp Gly Gly Leu Asn Lys Trp Glu Thr Asp Asp Asp Pro Trp
145 150 155 160

Ile Gln His Ala Arg Trp Phe Asp Lys Cys Asp Tyr Val Lys Leu Val
165 170 175

Lys Gly Lys Asp Phe Ile Gln Lys Val Met Thr Gln Ser Thr Phe Ile
180 185 190

Lys Ser Ser Lys Lys Glu Asn Ile Pro Glu Ile Asn Ile Ser Asn Asp
195 200 205

Glu Lys Asn Asp Ile Lys Leu Cys Lys Ile Cys Tyr Ile Glu Glu Arg
210 215 220

Val Ile Cys Phe Val Pro Cys Gly His Ile Phe Cys Cys Gly Lys Cys
225 230 235 240

Ala Ile Ser Met Asp Lys Cys Pro Val Cys Arg Asn Lys Ile Lys Asn
245 250 255

Leu Thr Arg Val Tyr Tyr Pro
260

<210> 16

<211> 295

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 16

Met Asn Phe Met Pro Gln Tyr Tyr Tyr Ile Ser Asp Ile Asn Asn Glu
1 5 10 15

Ile Glu Tyr Asp Glu Asn Phe Asn Pro Gly Lys Lys Phe Asp Phe Lys
20 25 30

Arg Gln Gly Gln Ile Lys Leu Leu Met Asn Glu Ile Arg Phe Leu Thr
35 40 45

Glu Asp Val Glu Leu His Lys Asn Tyr Lys Asn Glu Asn Ile Asn Ile
50 55 60

Leu Tyr Ile Gly Ser Gly Lys Gly Tyr His Ile Pro Leu Leu Ile Asn
65 70 75 80

Met Tyr Ser Asp Tyr Lys Ile Gln Trp Asp Leu Tyr Asp Pro Cys Gly
85 90 95

His Cys Glu Lys Leu Tyr Asn Ile Gln Lys Asn Asn Asn Asn Ile Lys
100 105 110

Ile Tyr Asp Thr Tyr Phe Asn Lys Ser Asp Val Glu Lys Tyr Glu Asn
115 120 125

Ile Asp Asn Leu Leu Phe Ile Thr Asp Ile Arg Thr Val Asp Asn Pro
130 135 140

Asp Asp Glu Pro Asn Thr Lys Asn Leu Ile Asn Asp Tyr Glu Leu Gln
145 150 155 160

Asn Tyr Ile Leu Lys Glu Leu Lys Pro Ile Ser Leu Val Lys Gln Arg
165 170 175

Asp Pro Phe Pro Asn Asp Trp Asp Asp Ser Tyr Lys Leu Ser Ile Pro
180 185 190

Asp Gly Lys Glu Tyr Ile Gln Cys Phe Gln Lys Tyr Asn Ser Ala Glu
195 200 205

Tyr Arg Ile Phe Ile Ser Gly Ile Thr Thr Phe Val Asp Ile Asn Ser
210 215 220

Val Ile Leu Asn Lys Arg Gly Ile Asp Arg Lys Leu Ala Trp Tyr Asn
225 230 235 240

Met Lys Tyr Arg Phe Gln Asn Asp Asn Asp Tyr Lys Ile Ala Tyr Arg
245 250 255

Ile Leu Asn Lys Tyr Ile Lys Ser Glu Asn Lys Pro Ile Leu Lys Lys
260 265 270

Tyr Asn Asn Ile Asn Lys Asn Asn Ile Lys Asn Val Ile Arg Ser Leu
275 280 285

Ser Lys Glu Met Gly Tyr Tyr
290 295

<210> 17

<211> 289

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 17

Met Asp Val Asn Lys Tyr Ile Tyr Glu Tyr Asn Lys Pro Leu Tyr Tyr
1 5 10 15

Thr Tyr Tyr Asp Leu Cys Arg Asn Met Asn Asp Val Ile Tyr Asp Tyr
20 25 30

Asn Asn Asn Thr Ile Lys Lys Tyr Met Asp Ile Leu Leu Ser Gln Ile
35 40 45

Gln Phe Leu Ser Asn Ile Asn Ile Lys Lys Cys Asn Asn Thr Asn Gly
50 55 60

Ile Val Asn Ile Leu Tyr Ile Gly Ser Ser Lys Ala Tyr His Phe Asn
65 70 75 80

Ile Leu Asn Glu Leu Tyr Lys Asn Leu Thr Asn Ile Gln Trp Tyr Phe
85 90 95

Tyr Asp Ile Ile Asp Pro Cys Ile Ser Val Glu Arg Leu Ser Tyr Asn
100 105 110

Ile Ile Phe Asn Arg Leu Phe Thr Glu Asp Asp Ile Ile Asp Phe Lys
115 120 125

Asp Lys Tyr Pro Leu Ile Leu Ile Tyr Asp Tyr Asp Asp Lys Ser Asn
130 135 140

Val Arg Asp Leu Leu Tyr His Tyr Asn Met Gln Asn Asn Ile Ile Ile
145 150 155 160

Tyr Leu Asn Pro Thr Tyr Ser Leu Leu Lys Phe Lys Tyr Met Pro Ile
165 170 175

Lys Trp Asn Asn Ser Phe Asn Asp Tyr Glu Tyr Ile Ser Thr Gly Ile
180 185 190

Lys Tyr Leu Pro Thr Ile Lys Ser Leu His Thr Arg Asn Ile Ile Asp
195 200 205

Asn Lys Asn Ile Met Thr Leu Thr Phe Asp Glu Ile Glu Ser Glu Asn
210 215 220

Tyr Tyr Glu Lys Met Asn Tyr Tyr Asn Asn Cys Gly Tyr Asn Asp Ile
225 230 235 240

Tyr Asn Asn Ile Ser Gly Tyr Ile Leu Asn Lys Ser Asn Leu Tyr Asp
245 250 255

Asn Asn Asn Ser Ala Tyr Asn Ile Leu Lys Ile Tyr Glu Lys Asn Ile
260 265 270

Ile Asn Thr Ile Asn Glu Asp Lys Ile Phe Arg Ser Lys Glu Lys Tyr
275 280 285

Ile

<210> 18

<211> 1089

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 18

Met Pro Phe Leu Gly Thr Gly Ile Leu Lys Phe Asp Ile Thr Gln Leu
1 5 10 15

Gln Asn Lys Glu Lys Gly Ser Asp Tyr Asn Ala Ile Arg Tyr Leu Lys
20 25 30

Arg Ile Leu Asn Lys Pro Cys Asp Asn Asp Asp Ile Leu Ile Pro Tyr
35 40 45

Asp Lys Leu Glu Ser Lys Glu Ile Asn Ile Ile Tyr Asn Trp Tyr Ile
50 55 60

Ile Lys Pro Ser Ser Leu Glu Gln Phe Ile Val Cys Lys Cys Lys Asp
65 70 75 80

Tyr Asp Thr Glu Glu Ile Ile Tyr Ile Leu Phe Asp Ile Tyr Glu Tyr
85 90 95

Phe Leu Cys Asp Tyr Glu Leu Ser Glu Ser Asn Thr Lys Leu Lys Asn
100 105 110

Ile Lys Asn Asn Ile Lys Tyr Lys Asn Ser Phe Asn Ser Ser Tyr Leu
115 120 125

Val Leu Glu Asp Tyr Lys Ile Ile Thr Asn Glu Val Asn Ile Gln Tyr
130 135 140

Tyr Tyr Asn Tyr Thr Glu Asp Ser Lys Ile Thr Leu Asn Asn Asn Asp
145 150 155 160

Leu Val Leu Phe Met Thr Pro Tyr Lys Ile Glu Lys Ile Tyr Ser Lys
165 170 175

Asn Ile Phe Ile Asn Gln Tyr Arg Trp Phe Tyr Val Leu Asn Asn Ile
180 185 190

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Glu Pro Ser Gly Ser Tyr Arg Ile Asn Met Asp Asn Met Gln Lys Ile
        195                 200                 205

Lys Thr Tyr Asn Lys Asn Lys Thr Ser Tyr Tyr Cys Lys Asn Pro Lys
    210                 215                 220

Leu Leu Phe Ser Asn Tyr Val Lys Ile Asp Lys Ile Pro Ala Ser Arg
225                 230                 235                     240

Val Ser Ile Asp Ile Glu Cys Gln His Phe Gly Glu Phe Pro Thr Ala
                245                 250                 255

Asn Lys Phe Pro Ile Ser His Ile Cys Ile Asp Trp Tyr Met Asp Lys
            260                 265                 270

Asn Thr Asn Pro Ile Lys Lys Ile Ile Thr Leu Ile Asn Tyr Glu Ile
        275                 280                 285

Ile Lys Asn Tyr Val Gly Lys Lys Asp Lys Phe Ile Tyr Thr Glu Val
    290                 295                 300

Asn Lys Leu Leu Asn Thr Asn Lys Val Tyr Ile Thr Ile Tyr Cys Thr
305                 310                 315                     320

Glu Lys Tyr Met Leu His Phe Val Leu Tyr Thr Leu Arg Gln Asp Phe
                325                 330                 335

Asp Tyr Val Leu Thr Tyr Asn Gly His Asn Phe Asp Phe Thr Tyr Ile
            340                 345                 350

Gln Arg Arg Lys Ile Asn Lys Leu Lys Gly Leu Cys Leu Asp Asn Val
        355                 360                 365

Tyr Ser Thr Asn Glu Ile Lys Ile Ser Lys Phe Ser Tyr Asn Gln Asp
    370                 375                 380

Thr Thr Tyr Glu Ile Asp Ser Thr Asn Gly Ile Ile Phe Leu Asp Leu
385                 390                 395                     400

Tyr Asn Tyr Ile Lys Lys Thr Tyr Pro Ser Ser Asn Tyr Lys Leu Ser
                405                 410                 415

Glu Ile Thr Lys Glu Arg Phe Asn Ile Phe Cys Lys Ile Ser Tyr Asn
            420                 425                 430

Asn Asn Glu Tyr Ile Ile Glu Pro Leu Asn Thr Lys Ala Asn Lys Asn
        435                 440                 445

Lys Ile Ser Ile Phe Tyr Asp Val Ile Arg Thr Ala Asn Tyr Cys Phe
    450                 455                 460
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Ile Asn Asn Asn Pro Tyr Lys Lys Asn Lys Thr Glu Ile Ile Asp Asp
465 470 475 480

Ile Glu Lys Leu Tyr Asp Leu Thr Ser Ile Lys Asn Ser His Asn Lys
485 490 495

Lys Phe Thr Ile Tyr Glu Asn Asp Ile Pro Ile Asn Asp Asn Tyr Ala
500 505 510

Thr Val Met Leu Ser Lys Asp Asp Val Asp Ile Gly Asp Lys Asn Ala
515 520 525

Tyr Val Phe Thr Lys Glu Lys Ser Asp Asn Ile Ala Tyr Tyr Cys Thr
530 535 540

His Asp Thr Val Leu Cys Asn Cys Ile Phe Lys Tyr Asp Met Ile His
545 550 555 560

Asp Lys Ile Ile Ala Phe Ser Asn Glu Tyr Leu Leu Pro Gln Cys Met
565 570 575

Ala Phe Lys Tyr Lys Ser Ser Asn Asn Ile Ser Gly Leu Leu Lys Thr
580 585 590

Leu Tyr Ser Asn Lys Thr Met Ile Tyr Pro Gly Asn Val Glu Phe Glu
595 600 605

Lys Phe Glu Gly Gly Tyr Val Ile Glu Pro Lys Gln Lys Tyr Ile Asp
610 615 620

Ser Leu Thr Ala Val Phe Asp Phe Asn Ser Glu Tyr Pro Ser Ile Ile
625 630 635 640

Ile Glu Ala Asn Leu Ser Pro Glu Val Val Lys Val Ile Lys Leu Phe
645 650 655

Asp Asp Glu Glu Ala Ala Asn Lys Val Glu Lys Tyr Leu Lys Asp Asn
660 665 670

Tyr Lys Tyr Pro Asp Tyr Cys Tyr Ile Lys Ile Ile Lys Asp Lys Met
675 680 685

Tyr Lys Phe Ile Leu Met Asp Arg Arg Glu Leu Gly Val Thr Thr Gln
690 695 700

Met Val Lys Gly Arg Glu Met Lys Asn Met Tyr Lys Asp Leu Lys Asn
705 710 715 720

Lys Asn Lys Asp Asn Val Asp Leu His Asn Phe Tyr Ser Ser Ala Leu
725 730 735

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Tyr Ser Lys Lys Ile Thr Ile Asn Ser Met Tyr Gly Leu Ser Gly Ser
            740                 745                 750

Glu Arg Phe Ile Phe Asn Ser Pro Tyr Cys Ala Glu Tyr Cys Val Gln
        755                 760                 765

Gly Gln Asn Cys Ile Lys Tyr Ile Gln Thr Leu Val Asn Asn Ser Lys
    770                 775                 780

Tyr Ile Asp Asn Val Leu Ile Leu Asn Lys Cys Asn Asn Pro Phe Thr
785                 790                 795                 800

Asn Glu Pro Ile Lys Thr Asn Tyr Pro Gly Asn Leu Asn Val Asn Phe
                805                 810                 815

Thr Phe Asn Val Lys Tyr Gly Asp Thr Ser Leu Phe Ile Thr Val Asn
            820                 825                 830

Phe Glu Ser Lys Phe Asn Ser Lys Glu Glu Lys Val Lys Val Gly His
        835                 840                 845

Lys Cys Phe Thr Phe Leu Gly Asn Val Ile Asn Asp Lys Lys Asn Lys
    850                 855                 860

Ile Leu Thr Asp Asn Phe Glu Phe Glu Tyr Glu Lys Met Tyr Tyr Trp
865                 870                 875                 880

Met Ile Leu Leu Lys Lys Lys Tyr Ile Gly Glu Val Val Ile Asn Met
                885                 890                 895

Asp Pro Leu Gln Leu Met Asp Asp Thr Lys Gly Thr Ala Leu Ile Arg
            900                 905                 910

Arg Asp Cys Thr Val Ile His Lys Thr Ile Leu Lys Asn Thr Ile Asn
        915                 920                 925

Ile Leu Lys Asp Phe Leu Thr Asn Asp Asn Thr Gly Ile Asn Ile Asn
    930                 935                 940

Val Lys Ile Asn Asp Tyr Leu Ser Ser Ala Phe Lys Asn Ile Ile Glu
945                 950                 955                 960

Asn Ile Gln Asn Leu Asp Ile Asn Asp Phe Lys Lys Ser Val Lys Tyr
                965                 970                 975

Ser Gly Val Tyr Lys Asp Pro Asn Tyr Pro Ile Glu Leu Cys Val Lys
            980                 985                 990

Glu Tyr Asn Leu Lys Asn Pro Asn  Asp Lys Ile Thr Lys  Gly Gln Arg
        995                 1000                 1005
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Phe Asp Phe Ile Tyr Ala His Lys Ile Asn Glu Trp Ser Lys Asp
1010 1015 1020

Lys Lys Trp Asn Ile Lys Tyr Thr Ile Asp Ile Ser Lys His Val
1025 1030 1035

Ile Ile Leu Glu Asp Tyr Leu Lys Asn Lys Asn Asn Tyr Arg Ile
1040 1045 1050

Cys Val Glu Lys Tyr Ile Lys Asp Ile Leu Ser Asn Leu Asp Gln
1055 1060 1065

Ile Ile Asn Asp Lys Asn Ile Ile Lys Asn Ile Asp Ile Met Leu
1070 1075 1080

Asn Ser Tyr Glu Pro Gln
1085

<210> 19

<211> 611

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 19

Met Asn Asp Ile Asp Lys Asn Asn Ile Leu Asn Asn Lys Tyr Ile Gly
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Phe His Thr Ile Lys Glu Tyr Leu Asp Lys Tyr Lys Cys Pro Leu Gln
20 25 30

Phe Phe Val Gly Ala Pro His Ser Tyr Gln Ser Thr Glu Tyr Leu Asn
35 40 45

Lys Ser Tyr Thr Gly Arg Thr Ile Phe Val His Ser Lys Tyr Val Gly
50 55 60

Asn Ile Ala Lys Asp Lys Asn Ser Val Ala Leu Arg Asn Ile Lys Lys
65 70 75 80

Glu Leu Leu Tyr Leu Gln Asn Met Glu Ile Asn Asn Ser Gly Thr Val
85 90 95

Val His Leu Ser Leu Tyr Tyr Asn Lys Asn Gln Glu Glu Ser Leu Lys
100 105 110

Tyr Val Ala Asn Glu Leu Asn Lys Phe Cys Lys Val Leu Asp Asn Ile
115 120 125

Asp Asn Asn Tyr Phe Asn His Ile Ile Phe Glu Thr Thr Asn Asp Ile
130 135 140

Arg His Leu Gly Ala Lys Thr Glu Asp Phe Lys Ile Leu Tyr Asp Asn
145 150 155 160

Leu Asp Ser Asn Ala Lys Lys Arg Ile Lys Phe Cys Ile Asp Thr Ser
165 170 175

His Ile Phe Val Thr Phe Tyr Asn Ile Asn Thr Val Lys Gly Met Ile
180 185 190

Asn Tyr Leu Ala Lys Phe Asp Leu Leu Ile Gly Leu Asp Lys Ile Ile
195 200 205

Leu Ile His Leu Asn Asp Ser Cys Gly Leu Pro Leu Ser Ser Tyr Lys
210 215 220

Pro His Glu Ala Ile Gly Lys Gly Asn Ile Phe Lys Asn Tyr Lys Asp
225 230 235 240

Asp Leu Ser Ser Leu His Ile Leu Lys Thr Tyr Ala Thr Leu Tyr Asn
245 250 255

Ile Pro Cys Ile Leu Glu Arg Arg Asn Glu Val Pro Asp Gln Ser Ile
260 265 270

Met Asp Glu Met Lys Ile Tyr Leu Asp Ile Lys Gln Asn Met Asn Ile
275 280 285

Asp Asn Phe Met Ser Met Ile Asn Lys His Lys Ile Leu Leu Val Leu
290 295 300

Asn Lys Phe Ala Asp Ile Tyr Asn Ile Leu Asn Glu Ile Lys Tyr Lys
305 310 315 320

Ala Phe Leu Asn Ala Ala Tyr Val Ile Gln Asn Thr Pro Val Ile Ile
325 330 335

Phe Lys Tyr Lys Asn Val Asn Asn Lys Phe Ile Leu Asn Glu Ser Lys
340 345 350

Glu Asn Ile Ile Gln Lys Tyr Lys Asn Leu Lys Ser Ile Gly Thr Ser
355 360 365

Ile Ser Asp Ile Ile Tyr Glu Leu Leu Ser Thr Asn Lys Val Glu Lys
370 375 380

Leu Ile Asn Leu Glu Asn Asn Ser Ser Tyr Lys Tyr Ile Lys Ile Leu
385 390 395 400

Thr Ser Ile Leu Phe Ile Gly Pro Lys Lys Ala Gln Ser Leu Leu Lys
405 410 415

Leu Asn Ile Lys Asn Ile Asn Asp Leu Ile Glu Lys Lys Asp Asn Ile
420 425 430

Ile Asn Met Gly Ile Leu Thr Ile His Glu Ile Lys Ile Ile Glu Tyr
435 440 445

Ile Lys Asp Met Glu Pro Val Ser Arg Asn Phe Ile Asn Asp Leu Lys
450 455 460

Gln Asn Ile Asn Leu Ser Ser Glu Cys Glu Trp Tyr Ile Leu Gly Ser
465 470 475 480

Tyr Ala Arg Gly Leu Asp Tyr Ser Lys Asp Ile Asp Ile Leu Ile Ile
485 490 495

Asp Phe Thr Ile Asp Lys Phe Leu Glu Glu Leu Lys Lys Ile Ala Lys
500 505 510

Leu Met Tyr Ile Ile Arg Lys Gly Asn Asn Ile Phe Ser Gly Val Phe
515 520 525

Leu Trp Gln Gly Lys Lys Phe Ile Leu Glu Ile Asn Lys Val Asn Asn
530 535 540

Lys Glu Lys Tyr Thr Ala Ile Met His Phe Thr Gly Ser Lys Lys Phe
545 550 555 560

Asn Ile Phe Met Arg Asn Ile Ala Lys Ser Glu Asn Met Ile Leu Asn
565 570 575

Gln Tyr Ser Leu Lys Lys Asp Asn Val Glu Leu Pro Ile Thr Lys Glu
580 585 590

Glu Asp Ile Phe Asp Tyr Leu Lys Ile Lys Tyr Ile Pro Asn Asn Lys
595 600 605

Arg Asn Ile
610

<210> 20

<211> 1381

<212> PRT

<213> Amsacta moorei entomopoxvirus

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Met Tyr Phe Asn Ile Leu Asn Gly Leu Leu Trp Lys Tyr Tyr Ile Ile
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Lys Arg Lys Lys Tyr Ile Tyr Asp Met Leu Glu Tyr Leu Leu Leu Ile
20 25 30

Leu Phe Phe Thr Leu Leu Tyr Ser Phe Lys Lys Asn Ile Lys Tyr Tyr
35 40 45

Asp Asn Asp Leu Asn Asn Ile Asn Lys Ile Asn Asn Asn Thr Asn Ile
50 55 60

Ile Tyr Tyr Pro Lys Ser Asn Ile Ser Ile Lys Ile Ile Glu Asn Val
65 70 75 80

Ala Lys Glu Leu Lys Ile Asn Lys Tyr Tyr Gly Ser Ser Asn Glu Asn
85 90 95

Glu Ile Ile Asn Phe Ile Asp Thr Asn Glu Thr Ile Phe Ile Leu Phe
100 105 110

Asn Asn Thr Cys Glu Asn Leu Leu Tyr Thr Ile Arg Phe Asn Asn Asn
115 120 125

Glu Asn Asn Asp Arg Leu Leu Ile Asn Ile Gln Trp Leu Ile Asn Met
130 135 140

Asn Tyr Leu Arg Leu Leu Ser Asn Lys Asn Ile Asn Ile Asp Ile Asp
145 150 155 160

Ile Asn Glu Tyr Ile Tyr Lys Asn Phe Asn Thr Asn Ile Leu Phe Tyr
165 170 175

Thr Tyr Tyr Ser Ile Leu Ile Ile Ala Phe Ile Ser Phe Ile Leu Lys
180 185 190

Asn Asn Asn Asp Asn Asn Asp Pro Met Phe Lys Ile Ile Lys Val Pro
195 200 205

Lys Ile Leu Ile Tyr Ile Ser Asn Phe Ile Cys Ser Ile Pro Phe Gly
210 215 220

Ile Ile Tyr Ser Val Phe Gly Thr Ile Ile Leu Thr Ile Ser Glu Asp
225 230 235 240

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Pro Leu Ile Asn Asn Asn Asn Asn Ile Ile Met Phe Leu Ile Leu Leu
                245                 250                 255

Ile Tyr Phe Ile Ser Val Ile Ser Met Ala Tyr Leu Asn Phe Phe Ile
            260                 265                 270

Leu Leu Ile Tyr Lys Tyr Lys Ile Phe Val Ile Met Cys Val Tyr Val
        275                 280                 285

Leu Thr Ile Ile Pro Ile Thr Leu Tyr Asn Asn Leu Asn Ser Asp Ile
    290                 295                 300

Asn Ile Phe Ile Gly Leu Ile Pro His Ile Pro Leu Tyr Trp Ile Phe
305                 310                 315                 320

Asp Gln Leu Asn Tyr Val Glu Lys Gln Asn Lys Ser Leu Thr Phe Asn
                325                 330                 335

Asn Asn Ile Ser Tyr Ser Ile Tyr Ser Lys Ser Ile Leu Ile Ser Ile
            340                 345                 350

Ile Tyr Leu Ile Leu Gln Ser Phe Ile Tyr Ile Ser Ile Ile His Ile
        355                 360                 365

Ile Lys Leu Ile Tyr Lys Ile Cys Lys Lys Tyr Met Lys Met Lys Tyr
    370                 375                 380

Ile Tyr Ile Ile Asn Glu Asn Asn Asn Tyr Met Leu Glu Thr Glu Asn
385                 390                 395                 400

Asn Asp Tyr Tyr Val Lys Ile Gln Asn Ile Tyr Lys Tyr Tyr Asp Asn
                405                 410                 415

Asn Phe Ile Leu Asn Asn Ile Cys Leu Asp Ile Ile Lys Asn Asn Thr
            420                 425                 430

Thr Val Leu Leu Gly Asn Asn Ser Ala Gly Lys Ser Thr Leu Leu Ser
        435                 440                 445

Ile Ile Phe Gly Leu Ile Lys Pro Asn Lys Gly Lys Ile Leu Thr Asn
    450                 455                 460

Asn Ile Lys Ile Gly Tyr Cys Pro Gln Asn Asn Ile Phe Thr Asp Phe
465                 470                 475                 480

Thr Val Lys Glu Asn Ile Tyr Leu Phe Asn Ile Leu Arg Gly Leu Ser
                485                 490                 495

Ser Leu Gln Ser Lys Ile Lys Thr Asn Glu Ile Ile Ile Tyr Leu Lys
            500                 505                 510
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Leu His Asp Ile Glu Asn Cys Ile Ile Thr Glu Leu Ser Glu Cys Ser
        515                 520                 525

Lys Arg Lys Leu Gln Leu Ala Phe Ser Leu Ile Asp Asp Ser Asp Phe
    530                 535                 540

Ile Leu Ile Asp Glu Pro Thr His Asn Ile Asp Leu Lys Ser Lys Gln
545                 550                 555                 560

Glu Ile Trp Asp Leu Ile Ser Leu Leu Lys Arg Asn Lys Thr Ile Leu
                565                 570                 575

Ile Thr Thr His Cys Ile Asp Glu Val Glu Leu Leu Ala Asp Asn Leu
            580                 585                 590

Ile Ile Leu Asn Asn Gly Asn Val Lys Tyr Asn Ser Thr Leu Phe Asn
        595                 600                 605

Ile Lys Lys Asp Ala Asn Val Thr Tyr Lys Leu Ser Ile His Asn Asn
    610                 615                 620

Ser Thr Asp Asp Lys Ile Lys Asn Ile Ile Ile Asn Ser Gly Phe Ile
625                 630                 635                 640

Ile Leu Asn Ile Asn Lys Ile Asp Glu Asn Asn Ser Ile Tyr Asn Ile
                645                 650                 655

Tyr Lys Thr Glu Asn Ser Asn Phe Leu Lys Leu Phe Glu Leu Leu Glu
            660                 665                 670

Asn Val Asn Cys Asp Ile Ile Tyr Phe Lys Ser Asn Thr Leu Asn Asp
        675                 680                 685

Ile Leu Tyr Lys Leu Cys Ser Glu Asp Ile Ile Ile Pro Asp Asp Ser
    690                 695                 700

Tyr Ile Asn Asn Leu Asn Tyr Asn Asp Met Phe Ile Ser Glu Ile Met
705                 710                 715                 720

Gly Phe Asn Lys Ile Met Arg Gln Phe Ile Glu Leu Phe Lys Arg Asn
                725                 730                 735

Ile Tyr Tyr Ile Arg Lys Asn Ile Leu Leu Phe Val Ile Ile Asn Phe
            740                 745                 750

Ile Leu Ser Ile Leu Ile Val Tyr Val Gly Ile Val Tyr Ile Lys Lys
        755                 760                 765

Tyr Glu Asn Leu Tyr Leu Tyr Asn Phe Val Ile Ile Asn His Asn Ile
    770                 775                 780
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Asp Asn Phe Ile Asn Asn Ser Asn Tyr Leu Leu Asp Ile Lys His Asn
785 790 795 800

Ser Thr Tyr Asn Lys Ile Thr Tyr Ile Pro Leu Phe Lys Tyr Ser Gly
805 810 815

Ser Ile Ala Ile Asn Ile Ile Ser Asn Ile Ile Ala Lys Ile Asn Ile
820 825 830

Pro Asn Ile Glu Lys Asp Ile Ile Thr Thr Ile Phe Tyr Pro Met Tyr
835 840 845

Gln Asn Lys Thr Ser Ile Leu Thr Asn Leu Phe Ile Ser Ile Ile Leu
850 855 860

Gln Leu Tyr Cys Ile Asn Tyr Asn Lys Leu Ile Lys Lys Asp Asn Ile
865 870 875 880

Asn Lys Thr Arg Lys Gln His Ile Ile Asn Gly Cys Asn Pro Glu Leu
885 890 895

His Trp Ile Thr Thr Leu Leu Phe Asn Met Ile Leu Phe Ser Ile Ser
900 905 910

Val Ile Pro Ile Ile Leu Tyr Met Leu Asn Ile Lys Ser Phe Phe Asp
915 920 925

Leu Ile Ile Leu Tyr Phe Ile Leu Ile Ile Asn Ala Leu Ser Phe Met
930 935 940

Leu Phe Ser Ile Ile Ile Leu Met Phe Asp Asn Gln Ser Asp Lys Ile
945 950 955 960

Ile Leu Ile Leu Val Phe Ile Leu Gly Ile Leu Leu Pro Ile Tyr Lys
965 970 975

Ile Lys Tyr Lys Asn Ile Ile Leu Asp Ile Leu Ser Tyr Ile Phe Ile
980 985 990

Pro Ser Cys Ile Ser Met Ser Ile Ile Glu Tyr Leu Asn Thr His Lys
995 1000 1005

Leu Asn Tyr Ile Ile Ser Ile Ile Ile Gln Ile Leu Leu Tyr Leu
1010 1015 1020

Ile Leu Ile Ile Leu Ile Glu Arg Gly Leu Ile Asp Ile Ile Tyr
1025 1030 1035

Asn Lys Ile Ile Asn Leu Lys Tyr Asn Arg Lys Asn Asn Asn Tyr
1040 1045 1050

Phe Glu Leu Gln Asn Ile Asn Lys Tyr Thr Asp Tyr Asn Ser Ser
1055 1060 1065

Leu Ile Met Ser Asn Val Tyr Lys Ile Tyr Asn Asn Lys Leu Ala
1070 1075 1080

Leu Asn Asn Ile Asn Phe Lys Ile Ser Glu Gly Lys Cys Phe Gly
1085 1090 1095

Ile Ile Gly Gly Asn Gly Cys Gly Lys Ser Thr Ile Phe Lys Ile
1100 1105 1110

Leu Ser Gly Glu Glu Cys Val Thr Lys Gly Asn Ile Tyr Ile Gly
1115 1120 1125

Cys Ser Asn Arg Ser Trp Ile Leu Lys Ser Asn Tyr Phe Lys Lys
1130 1135 1140

Ile Ser Tyr Cys Ser Gln Phe Phe Gly Ile Asp Thr Phe Leu Thr
1145 1150 1155

Gly Arg Gln Asn Leu Lys Leu Ile Met Ile Leu Asn Gly Phe Ser
1160 1165 1170

Asp Lys His Ile Gln Tyr Tyr Ile Asn Ile Trp Leu Lys Leu Leu
1175 1180 1185

Asn Ile Glu Lys Tyr Ala Asp Lys Ala Val Tyr Thr Tyr Ser Thr
1190 1195 1200

Gly Ile Ile Lys Arg Leu Lys Ile Ala Met Ser Leu Ala Pro Arg
1205 1210 1215

Ser Ile Leu Thr Leu Met Asp Glu Pro Thr Ser Gly Ile Asp Ile
1220 1225 1230

Val Ser Lys Gln Ile Ile Trp Lys Thr Ile Lys Tyr Ile Ile Asn
1235 1240 1245

Tyr Asn Tyr Tyr Asn Tyr Tyr Lys His Ser Ile Leu Ile Ser Ser
1250 1255 1260

Asn Asn Ile Glu Glu Ile Glu Tyr Leu Cys Ser Asn Val Ile Ile
1265 1270 1275

Leu Asp Ser Gly Asn Ile Met Tyr Asn Asp Thr Leu Glu Asn Ile
1280 1285 1290

Lys Asn Ile His Ser Thr Lys Ile Ile Asn Ile Lys Leu Leu His
1295 1300 1305

Tyr Asp Asn Asn Lys Ile Cys Lys Ile Lys Asn Lys Leu Lys Asn
1310 1315 1320

Lys Gly Phe Met Leu Lys Ser Asp Asn Lys Phe Lys Leu Thr Phe
1325 1330 1335

Cys Val Ser Lys Asn Ile Asn Leu Lys Tyr Ser Glu Leu Phe Lys
1340 1345 1350

Ile Leu Tyr Ile Leu Lys Asn Asn Tyr Ser Asp Ile Ile Asp Gln
1355 1360 1365

Tyr Asp Ile Ser Asp Thr Asn Ile Glu Gln Leu Phe Ser
1370 1375 1380

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<211> 79

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 21

Met Asn Tyr Tyr Ile Leu Leu Cys Leu Phe Met Leu Phe Ser Ser Ser
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Tyr Asn Phe Lys Leu Ile Asn Asn Asn Ile Cys Asn Glu Asp Tyr Asp
20 25 30

Pro Gly Ile Cys Arg Ile Gly Asp Ile Arg Trp Tyr Tyr Asn Tyr Asn
35 40 45

Ile Lys Asp Cys Lys Ile Phe Ile Tyr Gly Gly Cys Gly Gly Asn Met
50 55 60

Asn Asn Phe Asn Asn Tyr Glu Asp Cys Ile Asn Lys Cys Leu Ile
65 70 75

<210> 22

<211> 572

<212> PRT

<213> Amsacta moorei entomopoxvirus

<400> 22

Met Asn Ile Tyr Leu Lys Asn Ala Ser Asn Asp Thr Ile Ser His Leu
1 5 10 15

Ser Lys Phe Thr Asn Gln Ile Asn Asp Ile Ile Ser Phe Asp Ile Asn
20 25 30

Asn Phe Thr Lys Asn Val Leu Ile Met Arg Asn Asn Ile Asn Asn Ile
35 40 45

Arg Thr Asn Phe Glu Asn Val Ser Asp Asp Asn Ser Ile Lys Arg Arg
50 55 60

Ile Thr Glu Phe Phe Asp Lys Gln Asn Thr Pro Asn Leu Lys Leu Gly
65 70 75 80

Ser Ile Ile Ser Ile Ile Lys Phe Gln His Leu Thr Val Thr Tyr Val
85 90 95

Asn Lys Ile Ile Lys Glu Ile Val Thr Tyr Lys Cys Asn Thr Arg Glu
100 105 110

Ile Asn Ile Val Asn Phe Ser Ser Val Thr Ser Gln Ile Ser Asn Tyr
115 120 125

Asp Asn Pro Ile Leu Asn Glu Ile Leu Lys Gln Tyr Val Tyr Lys Gln
130 135 140

Lys Leu Lys Asn Val Thr Val Asn Asn Asp Lys Lys Lys Ile Ile Asp
145 150 155 160

Pro Asp Asp Glu Lys Leu Ala Glu Ser Ile Lys Lys Ile Leu Glu Glu
165 170 175

Ile Leu Lys Ile Leu Leu Ile Ile Lys Asn Asn Asp Cys Val Ala Tyr
180 185 190

Gly Ser Phe Thr Cys Tyr Asn Ile Asn Arg Ser Ile Lys Tyr Asn Asp
195 200 205

Ile Asp Leu Tyr Ser Thr Asp Ala Tyr Arg Ile Leu Ile Phe Phe Met
210 215 220

Ile Tyr Ile His Leu Thr Ile Gly His Asp Thr Cys Leu Phe Ser Ile
225 230 235 240

Pro Phe Ile Thr Gly His Ile Ser Leu Lys Tyr Lys Asn Ile Phe Ile
245 250 255

Ile Asp Cys Ile Phe Leu Asp Asn Ser Ile Ile Asn Val Ile Asn Lys

260 265 270

Ser Leu Ile Asn Asn Ile Tyr Phe Ile Asp Pro Gly Leu Gln Met Leu
275 280 285

Asn Asn Phe Arg Met Leu Ser Glu Asn Phe Arg Ser Tyr Lys Ile Tyr
290 295 300

Glu Lys Met Glu Glu Ser Leu Asn Lys Tyr Lys Thr Leu Leu Asn Tyr
305 310 315 320

Phe Val Asn Asn Asn Asn Lys Phe Asn Lys Gln Arg Leu Asn Tyr Trp
325 330 335

Leu Lys Ser Asp Val Cys Arg Asn Asn Phe Pro Tyr Thr Ile Val Asp
340 345 350

Asn Thr Ile Leu Ile Ser Ile Lys Glu Leu Ile Asp Ile Ser Pro Tyr
355 360 365

Asp Tyr Ile Met Ile Val Leu Asp Ser Pro Ser Asp Ile Met Glu Lys
370 375 380

Leu Ser Asn Ile Ser Gly Leu Phe Ser Arg Lys Tyr Gly Ala Phe Leu
385 390 395 400

Asn Glu Ile Phe Phe Glu Thr Lys Lys Ile Lys Asn Lys Ile Asn Thr
405 410 415

Tyr Ala Gly Asn Thr Asn Asn Ile Thr Gln Leu Ile Asp Glu Asn Lys
420 425 430

Leu Ile Lys Leu Asn Arg Ser Asp Ile Asn Met Pro Tyr Asn Ile Asn
435 440 445

Pro Asn Lys Lys Tyr Leu Ile Phe Ser Asn Leu Thr Thr Ser Thr Tyr
450 455 460

Val Tyr Phe Glu Asn Asp Lys Met Thr Asp Ile Ser Val Lys Asn Leu
465 470 475 480

Ile Ser Phe Ile Ser Thr Ala Cys Leu Tyr Asn Leu Leu His Lys Lys
485 490 495

Asp Asp Phe Gly Met Glu Leu Tyr Tyr Leu Thr Leu His Cys Leu Thr
500 505 510

Phe Thr Glu Thr Arg Lys Leu Asn Glu Tyr Lys Val Ile Asp Arg Tyr
515 520 525

Lys Ile Gly Glu His Lys Glu Ile Ser Leu Cys Lys Asn Leu Phe Asn

530 535 540

Ser Ile Tyr Lys Asn Lys Ser Met Glu Asp Glu Tyr Met Asp Tyr Asn
545 550 555 560

Thr Phe Ile Asp Leu Thr Asn Ile Asn Gly Gly Tyr
565 570

<210> 23
<211> 50000
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<213> Amsacta moorei entomopoxvirus

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